STUDIES OF SREBP CLEAVAGE IN ASCOMYCETOUS FUNGI BY THE DSC E3 LIGASE AND RBD2 RHOMBOID PROTEASE

by

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Abstract

Membrane-bound transcription factors have been shown to be activated by both regulated intramembrane proteolysis (RIP) and regulated ubiquitin-proteasome dependent processing (RUP). A classic example of a RIP-activated transcription factor is mammalian sterol regulatory element-binding protein (SREBP). SREBP is cleaved by the Site-1 and Site-2 proteases to release the soluble N-terminal transcription factor domain. In the fission yeast *Schizosaccharomyces pombe*, SREBPs are not cleaved via a RIP mechanism, because this organism lacks a Site-2 protease homolog, however the Golgi-localized Dsc E3 ligase has been shown to be required for SREBP cleavage in *S. pombe*. In this work, I explore the possibility that *S. pombe* SREBPs are cleaved by a novel combination of RIP and RUP, due to the identification of the Rbd2 rhomboid protease and a requirement for this enzyme in SREBP cleavage, and I show that the Dsc E3 ligase has conserved functions in the pathogenic fungus *Candida albicans*. I discuss the possibility of the Dsc E3 ligase and Rbd2 participating in Golgi protein quality control, and the potential use of the Dsc E3 ligase as a target for antifungal drugs. Together, these studies add to the knowledge of SREBP cleavage in ascomycetous fungi via the Dsc E3 ligase and the Rbd2 rhomboid protease.

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Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

Introduction

Sensing and responding to environmental stimuli is an important requirement for any cell to maintain homeostasis. Cells are constantly challenged with changing levels of a variety of molecules, including both intracellular and extracellular oxygen. Oxygen is required for many cellular processes, some of which are regulated by the availability of the O_2 molecule, so many labs have focused their studies on the transcriptional networks that control cellular adaptation to low oxygen. In this introduction, I will discuss examples of such transcriptional programs: those controlled by the sterol regulatory element-binding protein (SREBP) transcription factors in ascomycetous fungi. In particular, my work focused on the ascomycetes *Schizosaccharomyces pombe* and *Candida albicans*. Both of these organisms contain SREBP proteins, and both of these organisms use the SREBP transcriptional program to respond to environmental stimuli. However, in *S. pombe*, it has been known for almost a decade that the SREBP, Sre1, controls adaptation to low oxygen and low sterols, while in *Candida albicans*, relatively little is known about the transcriptional program governed by the SREBP, Cph2. The study of SREBPs led me to explore both regulated intramembrane proteolysis (RIP) and regulated ubiquitin-proteasome dependent processing (RUP). In this work, I discuss my discovery of a rhomboid protease involved in *S. pombe* SREBP activation, and my studies of SREBP activation in *Candida albicans*.

Transcription factor activation by RIP: The Classic SREBP Example and Classes of iCliPs

Cells need to mount a rapid and precise response to environmental stimuli, and to do this they orchestrate complex mechanisms to alter transcription of genes. This requires the activation of transcription factors, and within the last 20 years, a common theme has emerged of transcription factors activated by regulated intramembrane proteolysis (RIP) (Brown, Ye, et al. 2000). Many diverse processes are regulated by RIP, in organisms from bacteria to humans. RIP involves the regulated cleavage and release of a membrane-bound protein by an intramembrane protease. One classic example of RIP in transcription factor activation is the process by which cholesterol biosynthesis is regulated in mammals. Sterol Regulatory Element-binding Proteins (SREBPs) are a conserved class

of membrane-bound transcription factors found in many organisms from yeast to humans (Hughes, Todd and Espenshade 2005, Osborne and Espenshade 2009, Sakai, Duncan, et al. 1996, Brown and Goldstein 1997). Proteins in the SREBP family share a number of features. They are basic-helix-loop-helix leucine zipper (bHLH-LZ) transcription factors with their N- and C-termini located in the cytoplasm, and they contain two consecutive transmembrane helices separated by a short luminal loop. A mechanism for SREBP cleavage and activation has been well studied in mammalian cells (Figure 1.1) (Sakai, Duncan, et al. 1996, Osborne and Espenshade 2009). Mammalian SREBPs are synthesized in the ER and form a stable complex with SREBP Cleavage Activating Protein (Scap) (Hughes, Todd and Espenshade 2005, Hua, et al. 1996). Scap binds the C-terminus of the SREBPs, and when cells become depleted of cholesterol, Scap transports SREBPs to the Golgi. Once in the Golgi, two cleavage events occur, resulting in the release of the SREBP N-terminal domain from the membrane. The proteases responsible for these sequential cleavage events are the Site-1 Protease (S1P) and Site-2 Protease (S2P) (Rawson, et al. 1997, Duncan, et al. 1997, Cheng, et al. 1999, Sakai, Rawson, et al. 1998). S1P, a serine protease with its catalytic activity in the Golgi lumen, cleaves in the luminal loop, and this cleavage is required for SREBPs to access S2P, an intramembrane Zn-metalloprotease that cleaves SREBPs in the first transmembrane domain to release the N-terminus to the cytosol. S2P catalyzes the RIP event, allowing the membrane-bound transcription factor to thus be activated.

SREBP is only one example of RIP, and S2P is only one type of intramembrane protease. Nature has devised a variety of mechanisms to accomplish intramembrane proteolysis, and these intramembrane cleaving proteases are collectively known as iCliPs (Lal and Caplan 2011, Morohashi and Tomita 2013). iCliPs consist of metalloproteases (including S2P), aspartyl protease-like proteases (including presenillin-type and signal peptide peptidase-type), and serine proteases (the rhomboid proteases); the latter will be discussed in detail below. The example of SREBP illustrates a number of features common to RIP and iCliPs. These features are compartmental segregation, a priming cleavage event, and cleavage of a transmembrane substrate. The first common feature, segregation of protease and substrate in separate subcellular compartments, prevents inappropriate cleavage from occurring. This feature is especially important for the

rhomboid proteases, as they do not require a priming cleavage event like the other classes of iCliPs. The second feature, the priming cleavage event, is a second "checkpoint" in the proper delivery of the substrate to the protease. This cleavage event shortens the non-cytosolic portion of the protein to less than 30 amino acids, and appears to be a pre-requisite for iCliPs of the metalloprotease and aspartyl-like protease classes. The last feature, cleavage of a transmembrane substrate, is again common to all iCliPs. However, not all iCliPs cleave at the same region of the transmembrane segment (cytosolic versus non-cytosolic face), or the same type of transmembrane segment. Examples can be found where iCliPs cleave type-I, type-II, and multi-pass transmembrane proteins (Brown, Ye, et al. 2000, Ha, Akiyama and Xue 2013). Rhomboid proteases, again, are distinct from other iCliPs in this area, as they can also cleave juxtamembrane regions (Fleig, et al. 2012, Ha, Akiyama and Xue 2013). The variety and complexity of RIP by iCliPs is stunning, and represents a major mechanism by which membrane-bound transcription factors are activated.

Chewed up and spit out: Regulated ubiquitin-proteasome dependent processing

Another, less-well-studied mechanism for the activation of membrane-bound transcription factors has been described for several proteins. The group that first described this mechanism was initially perplexed by their results, as they had been studying an E3 ubiquitin ligase protein that was essential in the budding yeast *Saccharomyces cerevisiae* called Rps5, and found that the phenotype of an *RPS5* mutant could be suppressed by the truncated forms of two transcription factors (Hoppe, Matuschewski, et al. 2000). Through careful genetic and biochemical evaluation, they described a mechanism that would come to be known as regulated ubiquitin-proteasome dependent processing, or RUP. The canonical use of the ubiquitin-proteasome system (UPS) is to degrade aberrant proteins, so it came as a surprise that the Spt23 and Mga2 transcription factors were processed from their membrane-bound precursor forms to their soluble active forms by the action of this system, instead of being completely degraded (Hoppe, Rape and and Jentsch 2001, Hoppe, Matuschewski, et al. 2000). In the case of these two transcription factors, which are both anchored C-terminally in the ER membrane, ubiquitination through the action of the Rps5 E3 ligase causes recruitment of UPS machinery, including the 26S proteasome, to the protein. The transcription factor

then undergoes a processing event that degrades only the C-terminal portion of the protein, leaving the N-terminal transcription factor domain intact and available to translocate to the nucleus and upregulate transcription of the *OLE1* target gene. *OLE1* encodes the Δ9 fatty acid desaturase enzyme, and it was found that the Rps5-dependent RUP was regulated by levels of unsaturated fatty acids, similar to the regulation of SREBP RIP by cholesterol. This novel RUP mechanism described in *S. cerevisiae* was found to also exist in mammalian cells: the NF-κB pathway uses RUP to generate the active p50 subunit of this transcription factor via processing its p105 cytosolic precursor. Again, the C-terminal portion of p105 is degraded, leaving the N-terminal p50 protein intact to perform its cellular functions (Hoppe, Rape and and Jentsch 2001). The Spt23, Mga2, and p105 proteins share structural features, suggesting a common mechanism between these RUP substrates.

SREBPs in Fungi: Activated by RIP or RUP?

The fission yeast *Schizosaccharomyces pombe* has two SREBPs, Sre1 and Sre2 (Cheung and Espenshade 2013, Hughes, Todd and Espenshade 2005, Lloyd, Raychaudhuri and Espenshade 2013, Stewart, et al., 2011) (Figure 1.2 A). Sre1 is most similar to the mammalian SREBPs because it contains the C-terminal Scap-binding domain, and because it undergoes regulated trafficking and subsequent cleavage to release its N-terminus in response to low sterol levels. Hughes et al. (2005) showed that Sre1 activation also occurs during hypoxia, and that Sre1 target genes include those required for the hypoxic response. The second fission yeast SREBP, Sre2, lacks the C-terminal Scap-binding domain, and undergoes constitutive trafficking and cleavage to release its N-terminus (Stewart, et al., 2011, Cheung and Espenshade 2013). In the fungal pathogens *Cryptococcus neoformans* and *Aspergillus fumigatus*, SREBPs are key hypoxic transcription factors required for host adaptation and virulence (Chang, et al. 2007, Willger, Puttikamonkul, et al. 2008, Chun, Liu and Madhani 2007, Grahl and Cramer 2010, Willger, Cornish, et al. 2012). The fungal pathogen *C. albicans* has an SREBP homolog called Cph2 (Lane, et al. 2001), but known SREBP functions such as virulence, the hypoxic response, and regulation of sterol homeostasis do not require *CPH2*. However, *C. albicans* host intestinal colonization requires Cph2, suggesting that

Cph2 controls an undefined transcriptional program essential for commensalism (Rosenbach, et al. 2010).

The regulatory mechanisms behind SREBP cleavage and activation in ascomycetous fungi are unknown. In mammalian cells and the basidiomycete *C. neoformans*, SREBP cleavage occurs through the action of S1P and S2P (Shao and Espenshade 2012, Bien and Espenshade 2010), demonstrating a conserved RIP mechanism. However, no clear S1P and S2P homlogs can be identified in ascomycetous fungi (*S. pombe*, *A. fumigatus*, *C. albicans*), indicating that their SREBPs are cleaved by an alternative mechanism. In 2011, Stewart et al. identified several genes required for SREBP cleavage in *S. pombe* using a genetic screen of the non-essential haploid deletion collection (Kim, et al. 2010, Stewart, et al., 2011). These genes were termed Defective for SREBP Cleavage (*dsc*), and were shown to encode a Golgi-localized ubiquitin E3 ligase complex. The Dsc E3 ligase currently consists of six members: Dsc1-Dsc5 and Dsc6/Cdc48. Mutating each of the components of the complex disrupts cleavage of Sre1 and Sre2 (Figure 1.2 B). Dsc1 is a RING-domain containing protein, Dsc2 is a rhomboid pseudoprotease and the homolog to mammalian UBAC2, Dsc3 has a UBL domain, Dsc4 is a conserved fungal protein with no known domains, and Dsc5 contains a UBX domain that binds to Cdc48. The Dsc E3 ligase requires the activity of Ubc4 (an *S. pombe* E2 ubiquitin-conjugating enzyme) and the proteasome to carry out its SREBP cleavage function (Stewart, et al., 2011). The Dsc E3 ligase resides in the Golgi, and immunoprecipitation experiments show that Dsc2 can be co-purified with Sre2, suggesting that the complex interacts with SREBPs that have been trafficked to the Golgi (Lloyd, Raychaudhuri and Espenshade 2013, Stewart, et al., 2011). The proteasome was also identified as having an effect on Sre1 cleavage, although it was unclear if this effect was direct or indirect (Figure 1.2 C). Taken together, this information led to a model for Dsc-dependent SREBP cleavage where the SREBPs trafficked from the ER to the Golgi; in the Golgi SREBPs interacted with the Dsc E3 ligase, and cleaved in a proteasome-dependent manner to release the N-terminus (Figure 1.2 D).

When other fungal species were examined for the presence of the Dsc E3 ligase, it was found that several of them contained Dsc homologs, and functional studies in *A. fumigatus* demonstrated that the Dsc E3 ligase genes were required for cleavage of the

SREBP in that organism, SrbA (Willger, Cornish, et al. 2012). Interestingly, the Dsc E3 ligase is also conserved in *Saccharomyces cerevisiae*, an organism that lacks SREBPs (Ryan, et al. 2012). Because of this, it has been proposed that the Dsc complex may have broader functions in Golgi protein degradation (Stewart, et al., 2011, Lloyd, Raychaudhuri and Espenshade 2013, Ryan, et al. 2012).

Rhomboid Proteases: Conserved, yet Enigmatic Proteolytic Enzymes

The identification of the Dsc E3 ligase was the first time *S. pombe* SREBP cleavage inhibition had been shown, but questions arose regarding the exact mechanism of cleavage. The requirement for an E3 ligase and other UPS components such as Cdc48 and Ubc4 in *S. pombe* SREBP cleavage was suggestive of a RUP mechanism, rather than the RIP mechanism that is used by mammalian cells and *C. neoformans*. However, experiments similar to those used by Hoppe et al. to demonstrate a direct relationship between proteasome activity and conversion of *S. pombe* SREBPs from precursor to cleaved product were inconclusive (Espenshade lab, unpublished data). Equally unsuccessful were the attempts at identifying an ubiquitinated species of SREBP, so mechanisms other than RUP were reconsidered. If the *S. pombe* SREBPs were not cleaved via a RUP mechanism, it was unlikely that the Dsc E3 ligase, together with the proteasome, was responsible for the entire cleavage process. A yet undiscovered protease may be involved, much like the two-step mechanism for mammalian cleavage. Two separate genetic studies of SREBP cleavage resulted in the identification of a candidate gene encoding a rhomboid protease, later to be called *RBD2*.

Rhomboid proteases are a conserved family of serine proteases, present in almost all organisms (Freeman 2008, Ha, Akiyama and Xue 2013, Urban 2006, Urban 2010, Urban and Wolfe 2005). They are unique from soluble serine proteases because their catalytic residues are submerged within the plane of the membrane, and because they use a Ser-His catalytic dyad instead of the conventional Ser-Asp-His catalytic triad. The core rhomboid protease domain has six transmembrane helices arranged to yield a hydrophilic internal cavity where substrate cleavage can occur (Ben-Shem, Fass and Bibi 2007, Ha, Akiyama and Xue 2013, Vinothkumar, et al. 2010). There are variations on this theme, however, leading to the classification of rhomboid proteases into three major subtypes

(Strisovsky 2013, Kinch and Grishin 2013). The first subtype is the PARL-like group of rhomboids, which have one additional transmembrane helix preceding the core domain (1+6 topology). This subtype includes the only characterized fungal rhomboid, Rbd1/Pcp1 from *S. cerevisiae*. Most members of this group localize exclusively to the mitochondria. The second subtype is the secretase A group of rhomboids, which have an additional transmembrane helix at their C-terminal end (6+1 topology), and includes the well-characterized prokaryotic rhomboids GlpG and AarA. The last subtype is the secretase B group of rhomboids, which retain only the core six transmembrane helices, and include the mammalian RHBDL4 and the yeast Rbd2 rhomboids, which will be discussed at length in Chapter 2. Secretase rhomboids of both the A and B groups localize to organelles of the secretory pathway, and are not found on mitochondrial membranes.

Rhomboids catalyze cleavage of transmembrane helices of substrate proteins, although several examples of cleavage juxtamembrane regions have been shown. They cleave transmembrane helices with little apparent sequence specificity (Akiyama and Maegawa 2007, Maegawa, Ito and Akiyama 2005, Maegawa, Koide, et al. 2007, Urban and Freeman 2003). Instead, rhomboids have structural requirements for cleavage: transmembrane substrates of rhomboids generally have helix-breaking residues near the site of cleavage such as glycine, proline, tryptophan, and asparagine, and several studies have found structural features of the amino acid side chains as important requirements for the P4, P2, and P2' residues in the substrate. Because of these minimal, loosely conserved structural requirements, identification of rhomboid substrates has been challenging, and requires experimental identification, rather than a bioinformatic approach. Rhomboid proteases have so far been shown to function in cellular processes such as signaling and microbial pathogenesis (Freeman 2008); only one fungal rhomboid has been characterized, *S. cerevisiae* Rbd1/Pcp1, which localizes to mitochondria (Hill and Pellegrini 2010, McQuibban, Saurya and Freeman 2003).

S. pombe SREBP cleavage: Implications for Treating Infections of Opportunistic Fungal Pathogens

The Espenshade lab identified ORF *SPCC790.03* via two separate genetic

experiments that sought additional genes involved in SREBP cleavage (the specifics of these experiments will be discussed in detail in Chapter 2). Upon learning that this gene encoded a putative rhomboid protease, it became unclear whether *S. pombe* used RIP, RUP, or a combination of both proteolytic mechanisms to generate active SREBP transcription factors. The importance of studying the SREBP cleavage mechanism in *S. pombe* was now more important than ever, if not only to make contributions to the field of regulated proteolysis, but also to identify possible targetable mechanisms in treating infections caused by opportunistic fungal pathogens.

Several studies had already demonstrated the importance of SREBPs as virulence factors in opportunistic fungal pathogens such as *C. neoformans* and *A. fumigatus*, and had implicated a putative SREBP as an important factor in host intestinal colonization in *C. albicans* (Bien and Espenshade 2010, Chang, et al. 2007, Rosenbach, et al. 2010, Willger, Cornish, et al. 2012, Willger, Puttikamonkul, et al. 2008). Additionally, it had been shown that inhibition of the proteolytic activation of SREBPs in *C. neoformans*, which uses the canonical S2P RIP mechanism for cleavage, caused increased sensitivity to antifungal drugs, and decreased virulence in a mouse model of infection (Chang, et al. 2007, Bien and Espenshade 2010). One drawback to targeting the *C. neoformans* SREBP cleavage pathway is the similarity of the cleavage mechanism to mammalian cells. A constant challenge in the development of antifungal drugs is that fungal pathogens are eukaryotic cells – unlike trying to treat a bacterial infection, there are fewer physiological differences between the pathogen and host cells, and therefore drugs that target the pathogen are either weakly effective or have negative side effects on the patient. Understanding the mechanism of SREBP cleavage in *S. pombe* would translate into better understanding of SREBP cleavage in other ascomycetes, including the fungal pathogens *A. fumigatus* and *C. albicans*, and potentially allowing for the generation of antifungal therapies against these organisms.

Thesis Summary and Aims

When I began this work, the Espenshade lab had identified the Dsc E3 ligase as being required for SREBP cleavage and activation in *S. pombe*. Preliminary data suggested that other genes might also be required for this process, including an uncharacterized ORF *SPCC790.03*. For my thesis project, I aimed to (1) characterize ORF *SPCC790.03* and its gene product, the rhomboid protease Rbd2, (2) elucidate the relationship between Rbd2 and the Dsc E3 ligase, and (3) begin to characterize the *Candida albicans* Dsc E3 ligase and its relationship to Cph2. In doing so, I hoped to add to the mechanistic understanding of SREBP cleavage in ascomycetous fungi, which lack the S2P required for RIP of mammalian SREBP, and to contribute to the growing number of functionally characterized rhomboid proteases.

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Figure Legends

Figure 1.1: Mammalian SREBPs are Cleaved By RIP

Mammalian SREBPs are synthesized in the ER and form a stable complex with SREBP Cleavage Activating Protein (Scap). Scap binds the C-terminus of the SREBPs, and when cells become depleted of cholesterol, Scap transports SREBPs to the Golgi. Once in the Golgi, two cleavage events occur, resulting in the release of the SREBP N-terminal domain from the membrane. The proteases responsible for these sequential cleavage events are the Site-1 Protease (S1P), serine protease, and Site-2 Protease (S2P), an intramembrane Zn-metalloprotease. S1P cleaves in the luminal loop, and this cleavage is required for SREBPs to access S2P, which cleaves SREBPs in the first transmembrane domain to release the N-terminus to the cytosol.

Figure 1.2: The Dsc E3 Ligase Complex Cleaves Sre1 in Fission Yeast

A) SREBP proteins in *S. pombe*. Fission yeast contains two SREBPs, Sre1 and Sre2. Sre1 (left) more closely resembles mammalian SREBPs and contains the C-terminal Scap-interaction domain. Sre2 (right) lacks this C-terminal domain, but contains the conserved N-terminal bHLH-LZ transcription factor domain. Both proteins have two transmembrane domains separated by a short luminal loop, and have their cleavage sites predicted to be just outside the first transmembrane domain, in the cytosol. B) Figure from Stewart *et al.*, (2011). Western blot showing fission yeast Sre1 cleavage requires the *dsc1-dsc4* genes. Sre1 is cleaved in wild-type whole cell lysates after 6 hours of growth at $-O_2$, but only Sre1 precursor is present in whole cell lysates from *dsc* deletion strains (lanes 3-10). C) Figure from Stewart *et al.*, (2011). Western blot on whole cell lysates prepared from fission yeast grown at $-O_2$ for 0-4 hours. Wild-type yields a progressive accumulation of Sre1 N-terminus, while a proteasome mutant, *mts3-1*, has a severely reduced amount of cleavage. This experiment does not completely demonstrate RUP because the amount of precursor + cleaved Sre1 is not additive; the amount of precursor Sre1 decreases over time without an increase in the amount of cleaved Sre1. D) Model of SREBP cleavage in fission yeast (adapted from Stewart 2011). Sre1 is synthesized in the ER, and under conditions of low oxygen, Scap traffics with Sre1 to the Golgi. In the

Golgi, Sre1 interacts with the Dsc E3 ligase, Ubc4, and Cdc48, and the Sre1N transcription factor is released in a process that requires the proteasome. Sre1N can then go to the nucleus, bind SRE target sequences, and upregulate genes requires for hypoxic response.

Figure 1.1

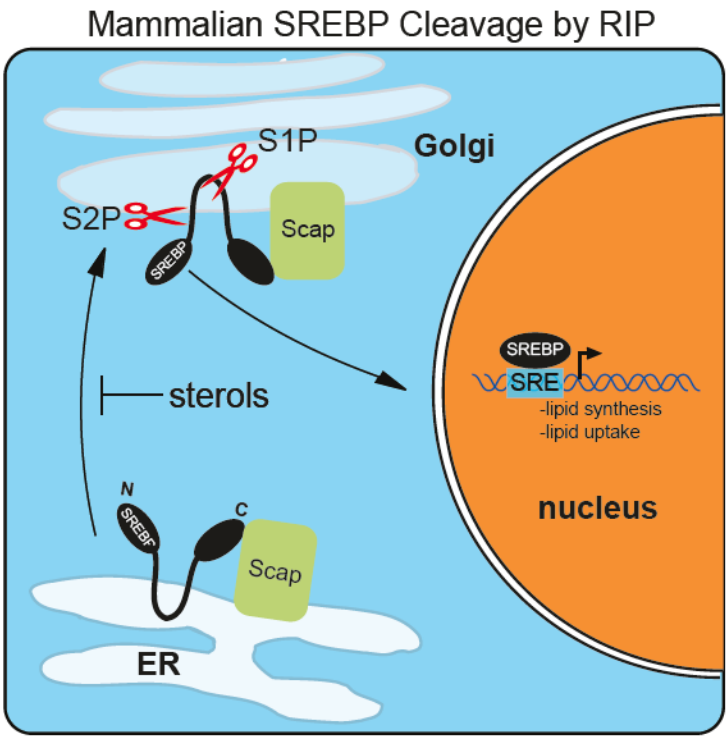
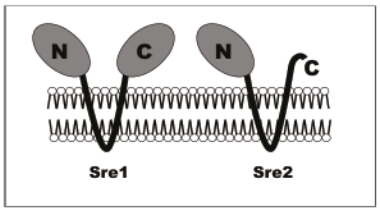

Mammalian SREBP Cleavage by RIP
S1P
Golgi
S2P
Scap
SREBP
sterols
SREBP
SRE
-lipid synthesis
-lipid uptake
nucleus
N
SREBP
C
Scap
ER

Figure 1.2

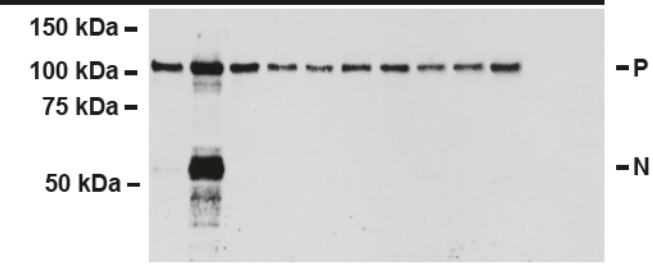
A



B

Western Blot - Deletion strains Anti-Sre1

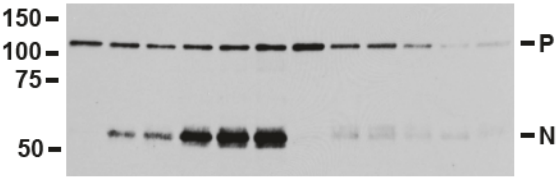
Strain	WT		*dsc1*Δ		*dsc2*Δ		*dsc3*Δ		*dsc4*Δ		*sre1*Δ	
6 hours +/-O_2	+	-	+	-	+	-	+	-	+	-	+	-
Lane	1	2	3	4	5	6	7	8	9	10	11	12



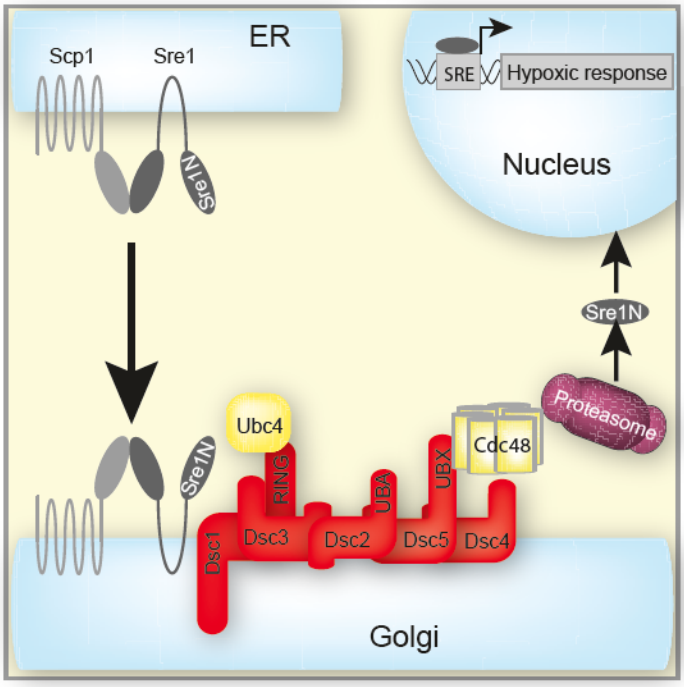
C

Western Blot - Anti-Sre1

Strain	WT						*mts3-1*					
Time -O_2 (h)	0	.5	1	2	3	4	0	.5	1	2	3	4
Lane	1	2	3	4	5	6	7	8	9	10	11	12



D



Chapter 2

Rbd2 rhomboid protease promotes fission yeast SREBP cleavage through interactions with Cdc48

Abstract

Sterol Regulatory Element-binding Proteins (SREBPs) are cleaved in the Golgi by the Site-1 and Site-2 proteases in mammalian cells, but in fission yeast, a Golgi E3 ligase complex called the Dsc complex facilitates cleavage in the absence of homologs of the mammalian proteases. Here, I present the characterization of a fission yeast rhomboid protease, Rbd2, and its role in SREBP cleavage. I identified *rbd2* through genetic interaction mapping, and I show that Rbd2 is located in the Golgi. An *rbd2Δ* strain is defective for SREBP cleavage, and SREBPs in this strain are degraded by the proteasome in a Dsc-dependent manner, suggesting the ubiquitination of SREBPs by the Dsc complex prior to the action of Rbd2. I show that Rbd2 can bind Cdc48 through a C-terminal SHP box motif, furthering a model in which the Dsc E3 ligase functions with protein quality control machinery to recruit Rbd2 to SREBP molecules to promote SREBP cleavage and activation.

Introduction

Conserved from yeast to humans, Sterol Regulatory Element-binding Proteins (SREBPs) are membrane-bound transcription factors responsible for adaptation to conditions of low sterols and, in fungi, low oxygen (Hughes 2005, Osborne 2009, Sakai, Duncan, et al. 1996, Brown 1997, Willger, et al. 2012). SREBPs have their N- and C-termini in the cytoplasm, two transmembrane domains separated by a short luminal loop, and their basic-helix-loop-helix leucine zipper (bHLH-LZ) transcription factors domain on the N-terminus (Rawson, et al. 1997, Duncan 1997, Cheng 1999, Sakai, Rawson, et al. 1998). The fission yeast *Schizosaccharomyces pombe* has two SREBPs, Sre1 and Sre2 (Cheung 2013, Hughes 2005, Lloyd 2013, Stewart, et al. 2011). Hughes *et al*. (2005) showed that Sre1 activation occurs in response to hypoxia, and Sre1 targets genes required for the hypoxic response. The second fission yeast SREBP, Sre2, undergoes constitutive trafficking and cleavage to release its N-terminus (Stewart, et al. 2011, Cheung 2013). Although SREBPs are conserved in fission yeast, they are cleaved by a mechanism distinct from mammalian SREBP cleavage.

The mechanism for *S. pombe* SREBP cleavage was partially worked out in a set of papers from Stewart et al. Using both a genetic screen of the non-essential haploid deletion collection and a genetic selection for genes required for hypoxic growth, they identified several genes required for SREBP cleavage that they termed the Defective for SREBP cleavage (*dsc*) genes. The *dsc* genes were shown to encode a Golgi-localized ubiquitin E3 ligase complex. This complex currently consists of six members: Dsc1-Dsc5 and Dsc6/Cdc48. Mutating each of the components of the complex disrupts cleavage of Sre1 and Sre2. Dsc1 is a RING-domain containing protein, Dsc2 is a rhomboid pseudoprotease and the homolog to mammalian UBAC2, Dsc3 has a UBL domain, Dsc4 is a conserved fungal protein with no known domains, and Dsc5 contains a UBX domain that binds to Cdc48. The Dsc complex is conserved in *Saccharomyces cerevisiae*, an organism that lacks SREBPs. Because of this, it has been proposed that the Dsc complex may have broader functions in Golgi protein quality control (Stewart, et al. 2011, Lloyd 2013, Ryan 2012).

The Dsc complex is an E3 ligase complex that requires the activity of Ubc4 (an *S. pombe* E2 ubiquitin-conjugating enzyme) and the proteasome to carry out its SREBP

cleavage function (Stewart, et al. 2011). It resides in the Golgi, and immunoprecipitation has shown that Dsc2 can be co-purified with Sre2, suggesting that the complex interacts with SREBPs that have been trafficked to the Golgi (Lloyd 2013, Stewart, et al. 2011). The identification of the Dsc complex was the first time *S. pombe* SREBP cleavage inhibition had been shown, but questions arose regarding the exact mechanism of cleavage. Due to the absence of a protease, it was unlikely that the Dsc complex, together with the proteasome, was responsible for the entire cleavage process, and a yet undiscovered protease may be involved, much like the two-step mechanism for mammalian cleavage. Based on SREBP cleavage in the mammalian system, this unknown protease could potentially be an intramembrane protease, of which there are four classes: metalloproteases of the S2P family, aspartyl proteases of the presenillin family, signal peptide peptidase, and rhomboid proteases (Wu 2006).

Rhomboid proteases are a conserved family of serine proteases, present in almost all organisms (Freeman 2008, Ha 2013, Urban 2006, Urban 2010, Urban and Wolfe 2005). Rhomboid proteases are unique from other serine proteases because their catalytic residues are submerged within the plane of the membrane, and because they use a Ser-His catalytic dyad instead of the conventional Ser-Asp-His catalytic triad. Rhomboid proteases have six transmembrane helices, arranged to yield a hydrophilic internal cavity where substrate cleavage can occur (Ben-Shem 2007, Ha 2013, Vinothkumar 2010). They are thought to catalyze cleavage of transmembrane helices of their substrate proteins, although several examples of cleavage juxtamembrane regions have been shown. These proteases cleave transmembrane helices with little apparent sequence specificity (Akiyama Y 2007, Maegawa, Ito and Akiyama 2005, Maegawa, Koide, et al. 2007, Urban and Freeman 2003) . Instead, they have structural requirements for cleavage: transmembrane substrates of rhomboids generally have helix-breaking residues near the site of cleavage such as glycine, proline, tryptophan, and asparagine. Because of this, identification of rhomboid substrates has been challenging, and requires experimental identification, rather than a bioinformatic approach. Rhomboid proteases have so far been shown to function in cellular processes such as signaling and microbial pathogenesis (Freeman 2008); only one fungal rhomboid has been characterized, *S. cerevisiae* Rbd1/Pcp1, which localizes to mitochondria (Hill 2010, McQuibban 2003).

Using a high-throughput genetic interaction method, I identified an *S. pombe* rhomboid protease as a new component of the SREBP cleavage pathway. Sequence alignment and conserved domain searches indicate that this protease is the homolog of *Saccharomyces cerevisiae RBD2,* and that the *S. pombe* Rbd2 rhomboid protease contains all of the conserved rhomboid protease elements. An *rbd2Δ/Δ* strain fails to cleave SREBPs, which are then degraded in a proteasome- and Dsc-dependent manner. *In vitro* studies show that Rbd2 specifically binds Cdc48 through a conserved SHP box in its C-terminus. This work provides a novel example of a fungal Golgi rhomboid protease functioning in a signaling pathway.

Results

Identification of the rbd2 rhomboid protease

Prior to joining the Espenshade lab, they decided to collaborate with Adam Frost and the University of Utah to take an unbiased approach to identify additional genes involved in SREBP cleavage in *S. pombe*. They chose to use a high-throughput genetic interaction (GI) mapping method. The measurement of GIs has been shown to determine the extent to which gene functions rely on each other (Roguev 2008, Frost 2012). Briefly, pair wise GI scores were determined for a set of 1,297 G418-marked "array" strains, crossed against 579 nourseothricin-marked "query" strains. Adam Frost included the *dsc* genes and several *cdc48* point mutants in the query set for a GI mapping experiment (Frost 2012). The hierarchical clustering revealed genes whose functional signatures were similar to the *dsc* genes, including a gene encoding a putative rhomboid protease that had not been previously associated with *S. pombe* SREBP cleavage. This gene, ORF *SPCC790.03*, was highly correlated with the other *dsc* genes and with *sre2* (Fig 2.1 A), so I chose to study this gene in more detail. The absence of *sre1* from the cluster was likely due to the fact that the study was performed under normoxic conditions, a phenomenon also observed in a previous GI study (Stewart, et al. 2011), where *sre1* is known to be inactive. The GI mapping experiment also showed a putative Arf/GEF that clustered with the *dsc* genes, *sre2*, and *rbd2*; experiments performed with this strain are presented in Supplemental Figure 2.1. From western blots probed for Sre1 and Sre2, I determined that this gene likely had no specific role in SREBP cleavage, but may have a role in trafficking Sre2, therefore yielding the GI scores that clustered the Arf/GEF gene near *sre2*.

The ORF *SPCC790.03* encodes a putative rhomboid protease, homologous to the *rbd2* gene in *S. cerevisiae*. I entered the SPCC790.03/Rbd2 protein sequence into the homology prediction program Phyre2 (Kelley and Sternberg 2009) to ask whether *S. pombe* Rbd2 had structural similarity to rhomboid proteases. Indeed, Rbd2 was predicted to have the structure of a rhomboid protease, with six transmembrane helices, and a catalytic Ser-His dyad (Fig 2.1 B, 2.1 C). The catalytic residues aligned with those of the *E. coli* rhomboid GlpG, in the fourth and sixth transmembrane helices, respectively (Fig

2.1 C). Phobius, a hydropathy prediction program (Kall, Krogh and Sonnhammer 2005, Kall, Krogh and Sonnhammer 2004, Kall, Krogh and Sonnhammer 2007), showed Rbd2 as a 6-pass transmembrane protein, with the N- and C-termini in the cytoplasm, placing the catalytic residues closer to the luminal face of the membrane.

An rbd2Δ strain is defective for SREBP cleavage

The GI results indicated that *rbd2* participated in SREBP cleavage, so I made an *rbd2Δ* strain to test for Sre1 and Sre2 cleavage defects. The *rbd2Δ* strain was unable to grown on medium containing $CoCl_2$, a hypoxia mimetic (Hughes 2005), indicating a defect in the production of the soluble N-terminus of Sre1, Sre1N (Fig 2.2 A). This growth defect was rescued by transforming the strain with either empty vector or a plasmid containing Sre1N. Expression of Sre1N returned growth of the *rbd2Δ* strain to wild-type levels. The *rbd2Δ* strain was unable to generate cleaved Sre1 when cells were grown in the absence of oxygen (Fig 2.2 B). Moreover, there was a decrease or disappearance of the precursor form of Sre1 in the *rbd2Δ* strain upon shifting cells to minus-oxygen. This disappearance was specific to Sre1, as actin protein levels remained constant (Fig 2.2 B). I asked whether the catalytic residues of Rbd2 were important for SREBP cleavage by integrating a catalytically dead *rbd2 (rbd2 H182A)* gene into the *his3* locus of the *rbd2Δ* strain. This mutant failed to rescue the Sre1 cleavage defect of the *rbd2Δ* strain, while a wild-type *rbd2* gene restored Sre1 cleavage (Fig 2.2 C).

I surveyed a previous collection of mutant strains defective in Sre1 cleavage for the presence of *rbd2* alleles (E. L. Stewart 2012)(E. L. Stewart 2012)(E. L. Stewart 2012). These strains were isolated from a genetic selection for mutants that failed to activate an Sre1 reporter, and also could not grow on $CoCl_2$. Using linkage analysis with the *rbd2Δ* strain, I found five alleles of *rbd2* in this strain collection, and sequence-verified the point mutations present in these strains. All of the *rbd2* point mutants were defective for Sre1 cleavage (Table 2.1, Fig 2.2 D).

SREBP precursor is degraded in a proteasomal- and Dsc-dependent manner in rbd2Δ.

The disappearance of the Sre1 precursor form in the *rbd2Δ* strain was a novel phenotype that I had not observed with other Dsc mutants. Our lab wanted to ask whether

this disappearance was due to proteasomal degradation, so we assayed the presence of Sre2 in an *rbd2Δ mts3-1* double mutant. The *mts3-1* strain contains a temperature-sensitive mutation in the 19S proteasome regulatory cap (Gordon 2006). Growth at non-permissive temperature blocks proteasome function. I chose to use Sre2 because it is constitutively cleaved in a Dsc-dependent manner, allowing me to remove the added complications of low-oxygen growth (Cheung 2013). The *rbd2Δ* strain showed the same phenotype for Sre2 as it does for Sre1 where there is a marked decrease in precursor protein compared to wild-type (Fig 2.3 A (Sumana Raychaudhuri), lanes 1 and 2). The *rbd2Δ mts3-1* double mutant showed recovery of the precursor form of Sre2 at non-permissive temperature (Fig 2.3 A, lane 3), indicating that the Sre2 precursor was being degraded by the proteasome in the *rbd2Δ* strain. The *mts3-1* mutation, by itself, did not cause any major cleavage defect, but did stabilize the N-terminal cleavage product (Fig 2.3 A, lane 4). The absence of this product in the *rbd2Δ mts3-1* strain further indicated a cleavage defect was occurring in the *rbd2Δ* strain.

To confirm the cleavage defect in the *rbd2Δ* strain, I used a previously developed model substrate of Sre2 (Cheung 2013). This Sre2 model substrate (Sre2MS) was shown to have Dsc-dependent cleavage like both endogenous Sre1 and Sre2 protein, but had the advantage of being constitutively trafficked to the Golgi like Sre2 and having an easily detectable size change after cleavage like Sre1. I transformed Sre2MS into wild-type and *rbd2Δ* strains and assayed cleavage in three isolates of each transformation by western blot. I used an empty vector plasmid as a control for the transformation and specificity of the expressed protein on the western blot. The western blot showed that the wild-type strain converted the majority of Sre2MS from precursor form to cleaved form (Figure 2.3 B, lanes 4-6), while the *rbd2Δ* strain failed to generate any cleaved form. Unlike the endogenous Sre1 and Sre2, a small amount of precursor was retained in the *rbd2Δ* mutant (Figure 2.3 B, lanes 10-12). We also performed a similar experiment with a chemical inhibitor of the proteasome, bortezomib (Bz) on the strains expressing Sre2MS. Bz treatment caused an increase in both precursor and cleaved forms of Sre2MS in the wild-type strain, and an increase in the precursor form only in the *rbd2Δ* strain (Figure 2.3 C (Sumana Raychaudhuri)). This result further demonstrated the SREBP cleavage defect caused by deleting *rbd2*.

Because I observed a loss of SREBP precursor only in *rbd2Δ* and not in any of the other *dsc* mutant strains, I hypothesized that the Dsc complex may be required for the *rbd2Δ*-dependent precursor disappearance. Previous studies have indicated that the loss of any of the Dsc subunits dissociates the complex, rendering it non-functional (Lloyd 2013). I made an *rbd2Δ dsc1Δ* double mutant, and observed recovery of the Sre2 protein in this strain (Fig 2.4 A, lanes 1-4). This indicated that the Sre2 disappearance was both proteasome-dependent and Dsc-dependent. I repeated this analysis with *dsc2-dsc4*, performing a genetic epistasis test. I recovered Sre2 precursor in all tested *dscΔ-rbd2Δ* double mutants (Fig 2.4 A). From these results I determined that the Dsc complex acts before Rbd2 in the biochemical pathway to cleave SREBPs. Preliminary data indicated that some SREBP processing components, such as *ubc4* and *cdc48*, may also act upstream, but the role of these non-membrane components was less clear (Supplemental Figure 2.2). To ask whether the disappearance of SREBP precursor protein in *rbd2Δ* required complex formation or E3 ligase activity, I made a point mutation in the catalytic RING domain of Dsc1 (*dsc1 C634A*) (Stewart, et al. 2011). The catalytically dead Dsc1 also recovered Sre2 precursor protein in an *rbd2Δ* background, demonstrating that the precursor disappearance required RING E3 ligase activity (Fig 2.4 B). Genetic epistasis testing was also performed with a *cdc48* mutant allele (E352K mutation) and a temperature-sensitive allele of *ubc4* (Supplemental Figure 2.2). These results of these experiments suggested a role for ubiquitination in SREBP processing, but need to be repeated and verified before drawing solid conclusions.

Rbd2 localizes to Golgi

To investigate the cellular localization of Rbd2, I generated a construct expressing Rbd2 under control of a CaMV promoter and tagged with six copies of mCherry fluorescent protein (Rbd2-6X-mCherry). I performed co-localization with three different GFP-tagged Golgi proteins: Anp1 (*cis*-Golgi), Sec72 (late Golgi), and Dsc2. Previous studies co-localized Dsc2 with Anp1 in the Golgi (Stewart, et al. 2011), and my experiments showed that Rbd2 also localized to the Golgi (Fig 2.5 A). I observed co-localization between Rbd2 and Sec72, Rbd2 and Anp1, and Rbd2 and Dsc2. Taken

together, these results indicate that Rbd2 and the Dsc E3 ligase both have steady-state localization in the Golgi.

I wanted to ask whether Rbd2 was physically interacting with the Dsc E3 ligase to facilitate SREBP cleavage. Previously, it has been shown that the Dsc E3 ligase forms stable physical connections (Lloyd 2013), and because Rbd2 co-localized with Dsc2 in the Golgi and had a role in SREBP cleavage, I tested whether I could detect interactions between Rbd2 and the other Dsc E3 ligase complex members. I generated a chromosomally integrated strain where Rbd2 was fused to a C-terminal tandem affinity purification (TAP) tag, and used co-immunoprecipitation to ask whether I could detect any interaction with Dsc2. Dsc1-TAP (Stewart, et al. 2011) was used as a control. I found no Dsc2 associating with Rbd2 in the bound fraction, indicating no stable physical interactions were occurring (Fig 2.5 B).

Rbd2 binds Cdc48 through a C-terminal SHP Box

Although it did not interact with the membrane-bound Dsc complex subunits, Rbd2 was predicted to have a Cdc48-interacting motif at its C-terminus. This motif was identified using the Phyre2 homology search program (Kelley and Sternberg 2009), which found similarity between Rbd2 and the p97/Cdc48 cofactor, p47. A 17-amino acid stretch was identified as having conserved sequences and structural homology. This sequence contained the p47 SHP-box, a previously characterized sequence present in several other Cdc48 binding proteins, including mammalian Ufd1 and yeast Dfm1 (Fig 2.6 A) (Schuberth 2008, Sato 2006, Bruderer 2004)

I wanted to explore the potential interaction between Rbd2 and Cdc48, so I purified Rbd2 C-terminus fused to GST (Fig 2.6 A). I showed that the Rbd2 C-terminus could bind Cdc48 equivalently to the Dsc5 UBX domain *in vitro* (Fig 2.6 B). I generated several constructs to determine the region of binding, and found that deleting the amino acids predicted to contain the SHP box greatly reduced binding; deleting the half of the C-terminus containing the SHP box completely abolished binding (Fig 2.6 C). Point mutants in the SHP box demonstrated that two residues, G244 and G246, also completely abolished binding, verifying the presence of a SHP box (Fig 2.6 D (He Gu)).

To study the effect of Cdc48 binding *in vivo*, I generated strains where the C-terminus was truncated on the chromosome, and assayed the effect on SREBP cleavage. The ΔSHP truncation, which removed the eleven amino acids containing the SHP box, retained some Sre1 cleavage, while the Δ26 truncation, which deleted the 26 amino acids demonstrated to be absolutely required for Cdc48 binding *in vitro*, failed to express Rbd2 protein at levels comparable to WT (Fig 2.6 E, F). Taken together, these results suggest a correlation between the amount of Cdc48-Rbd2 binding and the amount of SREBP cleavage.

Discussion

GI mapping led to identification of rbd2

In this study, I identified a rhomboid protease, Rbd2, which facilitates SREBP cleavage in *S. pombe*. I found *rbd2* using a GI mapping experiment, where it was highly correlated with the genetic profiles of the *dsc* genes and *sre2*. GI mapping is a powerful method to identify genes that are involved in related pathways and cellular processes (Frost 2012, Roguev 2008, Ryan 2012). The correlations I observed stem from the analysis of over 1000 different genes in *S. pombe* in this particular GI study. This increases confidence that the *rbd2 – dsc – sre2* correlations are meaningful, because the GI scores of *rbd2* with each of the array genes had to be highly similar to those with the *dsc* and *sre2* genes to obtain the clustering and correlations observed in this study. The putative Arf/GEF gene that also clustered with the *dsc-sre2,-rbd2* group of genes had an Sre2 cleavage defect, although this was only tested once and should be repeated for verification. Further experiments are needed to determine if this gene has a direct or indirect role in Sre2 processing, but I hypothesize that the cleavage defect is indirect and due to altered protein trafficking in this strain. This rationale may also explain the lack of an Sre1 cleavage defect in the Arf/GEF deletion strain. It is likely that this gene clustered with the *dsc*, *sre2*, and *rbd2* genes in the GI mapping experiment due to its Sre2 cleavage phenotype.

Possible Rbd2 cleavage mechanism

Our characterization of Rbd2 revealed that it contains all the structural and sequence features of a rhomboid protease. Rhomboid proteases, including Rbd2, share a high degree of structural conservation. Rbd2 retains the classic 6-TM structure, and its catalytic Ser and His residues are located in the fourth and sixth transmembrane domains, respectively. Rbd2 exhibits a longer C-terminus that is not conserved among all rhomboids, but appears to be a feature present in several other eukaryotic rhomboids. Few rhomboids have been functionally characterized, especially in eukaryotic cells. Other known Golgi eukaryotic rhomboids include *Drosophila* Rhomboid-1, which is responsible for the cleavage and activation of the Spitz EGF signaling pathway (Lee

2001, Urban, Lee and Freeman 2001)(Lee 2001, Urban, Lee and Freeman 2001)(Lee 2001, Urban, Lee and Freeman 2001). Almost all characterized rhomboids play a role in signaling (Freeman 2008, Ha 2013); I have shown that Rbd2 plays a role in the hypoxic response signaling pathway.

The requirement of Rbd2 and its catalytic activity in the cleavage of SREBPs demonstrates that it functions in the SREBP signaling pathway. It is tempting to speculate that Rbd2 directly cleaves the SREBPs. There have been several cases of rhomboid proteases cleaving multi-pass transmembrane proteins (Ha 2013), although most of the characterized rhomboid substrates have been single-pass transmembrane domain proteins. Current data shows that Sre1 and Sre2 are cleaved in the cytosol in a juxtamembrane region of the first transmembrane domain to release the N-terminus (Stewart, et al. 2011, Cheung 2013). However, based on the topology of Rbd2 I predict that this protease would cleave on the luminal side of the membrane, creating a discrepancy between the known N-terminal transcription factor product and the potential rhomboid cleavage products. Previous data suggested a role for the proteasome in SREBP cleavage (Stewart, et al. 2011), and examples are present where the proteasome shows limited proteolysis to generate a "cleaved product" by a process termed regulated ubiquitin-proteasome dependent proteolysis (RUP), so it is possible that the SREBPs could undergo additional processing after a rhomboid cleavage event in order to generate the observed N-terminal transcription factor products. Another attractive feature of this model is that a two-step cleavage mechanism is common to many examples of regulated intramembrane proteolysis (RIP). Of the three classes of proteases that catalyze RIP events, two require a primary cleavage event to shorten the non-cytoplasmic side of the transmembrane segment that will undergo RIP. Compelling data from Cheung and Espenshade also shows that a model substrate of Sre2 contains many residues in the second transmembrane domain that, when mutated, block *dsc*-dependent cleavage, lending further credence to the proposed location of Rbd2 cleavage. Additional work is needed to find the substrate(s) of Rbd2 and characterize the SREBP cleavage mechanism.

Model of SREBP Cleavage in S. pombe

It is clear that *rbd2* has a role in SREBP cleavage because the *rbd2Δ* strain shows a cleavage defect. Unlike the other *dsc* genes, the *rbd2Δ* strain showed a disappearance of the Sre1 and Sre2 precursor proteins. I determined this was due to proteasomal degradation of the precursor, and that this degradation required the E3 ligase activity of the Dsc E3 ligase. The activity of the E2 Ubc4 may also be required for SREBP disappearance (Supplemental Figure 2.2), leading me to hypothesize that the Sre1 and Sre2 proteins are ubiquitinated by the Dsc complex. This ubiquitination event appears to happen before the SREBPs encounter Rbd2 in the Golgi, but it is unclear whether they are ubiquitinated in the ER or the Golgi. The exact site of ubiquitination remains to be determined, but this is an active question being addressed with *in vivo* methods. It has already been published that the Dsc complex interacts with Sre2 *in vivo*, further supporting the possibility that the Dsc E3 ligase complex ubiquitinates SREBPs. The precursor form of Sre2 that is recovered in the *rbd2Δ mts3-1* double mutant does not appear to have a shifted molecular weight indicative of the addition of ubiquitin, but inhibition of the proteasome is known to deplete the pools of free ubiquitin in the cell (Hanna 2003), which may explain the lack of modified protein in this strain. The Rbd2-Cdc48 interaction is intriguing in the context of possible ubiquitination of the SREBPs by the Dsc complex. Cdc48 can bind ubiquitin (Dreveny 2004, Schuberth 2008), and it is possible that this interaction helps localize Rbd2 to the Dsc-SREBP protein complex. Future studies are aimed at exploring how the Rbd2-Cdc48 interaction relates to SREBP cleavage.

Recently, another example of a rhomboid protease binding to Cdc48 was shown in mammalian cells. The mammalian rhomboid protease, RHBDL4, binds p97/Cdc48 through a VBM in its C-terminus (Fleig 2012). This bears striking similarity to the binding I observed between Rbd2 and Cdc48. RHBDL4 acts with ER-associated degradation (ERAD) machinery to facilitate the cleavage and degradation of misfolded proteins in the ER. I have speculated that the Dsc complex may be functioning in Golgi protein quality control, so Rbd2 provides an attractive parallel to RHBDL4 in this regard. Further studies will address the extent of the similarity between these two systems and

look at the possibility of a conserved role of rhomboid proteases in protein quality control.

The data from this set of experiments led me to revise our model for SREBP cleavage in *S. pombe*. Previously, it was shown that the Dsc E3 ligase complex resided in the Golgi, where it interacted with SREBPs to facilitate their cleavage. This action required the E2 Ubc4 and the AAA-ATPase Cdc48. Here, I expand the SREBP cleavage model to include the rhomboid protease Rbd2 (Fig 2.7). I hypothesize that the Dsc E3 ligase complex is "marking" the SREBPs with ubiquitin prior to the action of Rbd2. Cdc48 may be facilitating the recruitment of Rbd2 to the Dsc complex and Ub-SREBP substrates. Rbd2 acts to facilitate cleavage, and the SREBP substrates are then further processed to generate the active transcription factor product. Questions remain over which protease is responsible for generating this final cleaved product. The lab has not identified any additional proteases, despite using several genetic and biochemical methods, but has shown a role for the proteasome (Stewart, et al. 2011). It is possible that Rbd2 is the only protease in the system, but I do not exclude the possibility that a cytosolic protease is required to complete SREBP processing. I also cannot exclude the possibility that Rbd2 is required for clearing the C-terminal fragment left behind after cleavage; it is currently unknown what happens to the C-termini of SREBPs after the N-terminus is released from the membrane. Expressing and purifying Rbd2, along with potential substrates, and performing in vitro cleavage assays could answer many of these questions.

The work described here lays the foundation for further study of fungal Golgi rhomboid proteases. Characterizing Rbd2 homologs in other species, particularly those that lack SREBPs such as *S. cerevisiae*, may help elucidate new pathways that require rhomboid proteases in these organisms. Additionally, the Rbd2-Cdc48 binding, similar to the characterized mammalian rhomboid RHBDL4, may be widely conserved among other eukaryotic rhomboids. This study provides a new context to explore the connection between protein quality control and rhomboid proteases.

Materials and Methods

Materials – I obtained yeast extract, peptone, Luria Broth (LB) and agar from BD Biosciences; Edinburgh minimal medium (EMM) from MP Biomedical; Geneticin from GIBCO; clonNAT from Werner BioAgents; cobalt chloride from Sigma; oligonucleotides from Integrated DNA Technologies; alkaline phosphatase from Roche; HRP-conjugated, affinity purified donkey anti-rabbit and anti-mouse IgG from Jackson ImmunoResearch; prestained protein standards from New England Biolabs; bortezomib (Velcade) from LC Laboratories; MagneGST beads from Promega; Brefeldin A from Sigma; nonfat dry milk from Carnation.

Strains and media – Wild-type haploid *S. pombe* KGY425 or KGY461 and derived strains were grown to log phase at 30 °C in YES medium (5 g/L yeast extract plus 30 g/L glucose supplemented with 225 mg/L each of adenine, uracil, leucine, histidine, and lysine) or EMM plus supplements, unless otherwise noted. $CoCl_2$, when used, was added to media at a concentration of 50 µg/L.

S. pombe strains were generated using homologous recombination via standard molecular biology and genetic techniques (Bahler 1998, Alfa 1993, Guthrie 1991). Geneticin (100 µg/L) and clonNAT (100 µg/L) were used to select for the *kanMX* and *natMX* marker genes, respectively. Transformants were screened for homologous integration using PCR. A complete list of strains is described in Table 1.

Genetic Interaction Study – I used data from a previously published genetic interaction study (Frost 2012). Included in the query set for the study were the *dsc* genes and several alleles of *cdc48*. Genetic interactions were mapped as previously described (Frost 2012). Hierarchical clustering profiles were analyzed for genes that clustered near the *dsc* genes.

Antibodies and Immunoblotting – I obtained antisera to Sre1 (aa 1-260) and Sre2 (aa 1-426) by generating polyclonal IgG generated against the cytosolic N-termini of the fission yeast proteins as previously described (Hughes 2005). Whole cell lysates were

prepared for immunoblotting analysis as described previously (Hughes 2005), except in the case of Sre2. Lysates for Sre2 immunoblotting were prepared using glass bead lysis in NP-40 buffer, followed by 2 minutes of centrifugation at 20,000 x g. A BCA assay was performed on the supernatants, and an equal amount of protein (in µg) was treated with Alkaline Phosphatase. Denaturing buffer with βME and loading dye was added and samples were then run on SDS-PAGE.

Low Oxygen Cell Culture – Sre1 hypoxic cleavage assays were performed as described previously (Cheung 2013, Hughes 2005, Stewart, et al. 2011). Briefly, cells growing in log phase were centrifuged to remove oxygenated medium and resuspended in deoxygenated medium under anaerobic conditions inside a Ruskinn Invivo2 400 hypoxic workstation (Biotrace, Inc.). Cells were cultured inside the workstation for 0.5-4 hours, depending on the particular experiment, before being removed, centrifuged, washed with water, and flash-frozen as cell pellets. Cell pellets were then stored at -80°C.

Linkage Analysis – Strains were mated for two days on malt extract medium, then cells were subjected to random spore analysis by plating on YES and YES+$CoCl_2$ (Nurse Lab Handbook). Mating was scored as positive (+) or negative (-) based on growth on YES, and linkage was scored (+ or -) based on growth on YES+$CoCl_2$. YES (+), $CoCl_2$ (+) matings were indicative of unlinked genes, and YES, (+) $CoCl_2$ (-) matings were indicative of linked genes. Matings that failed to grow on YES were not scored and indicated a lack of mating.

Fluorescence Microscopy – Images were taken on a 3i Spinning Disk Confocal (Marianis/Yokogawa; Leica) in the Johns Hopkins Microscope Facility (http://www.hopkinsmedicine.org/micfac/Equipment/3iSDC.html) using lasers emitting at either 488 nm or 562 nm for GFP and mCherry signals, respectively. Images were converted to TIFF format and analyzed using ImageJ, including the Colocalization Finder Plug-in (Schneider 2012). Minimum and maximum pixel intensity values for each channel were adjusted until background was negligible, then these values were applied to all images from the same imaging session. To determine the outlined boarders of cells as indicated in figures, the background was increased until cell outlines were again visible,

then lines were hand-drawn to indicate the location of individual cells, and the outlines were superimposed on the low-background images.

Proteasomal Inhibition – I used bortezomib at a concentration of 1 mM to inhibit the proteasome and treated strains for 2 hours. In strains with an *mts3-1* proteasome mutation, the proteasome was inhibited by growth at non-permissive temperature (36 °C) for 2 hours.

In Vitro GST Binding Assay – The *in vitro* GST binding assay was performed as previously described (Lloyd 2013). Briefly, GST-fusion proteins were expressed in *E. coli*, and bacterial lysates were incubated with MagneGST beads. The beads were then washed before *S. pombe* cytosol was added to the reaction. Boiling with a buffer containing SDS and βME eluted bound proteins.

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Figure Legends

Figure 2.1: Identification and bioinformatic analysis of SPCC790.03.

A) Using a large-scale genetic interaction technique, I identified the ORF *SPCC790.03* as having high correlation scores with the DSC complex members *dsc1-dsc4* and *sre2*. On the x-axis, correlation scores with *sre2* for all tested genes are plotted. *dsc* genes and *SPCC790.03* have similar correlation scores with *sre2*. On the y-axis, correlation scores with *SPCC790.03* for all tested genes are plotted. *dsc* genes and *sre2* have similar correlation scores with *SPCC790.03*. Overall, the *dsc* genes, *sre2*, and *SPCC790.03* form a cluster with higher-than-average correlation scores. B) *SPCC790.03* encodes a previously uncharacterized rhomboid protease. The protein predicted from this gene has the characteristic 6-TM structure of a rhomboid protease, as shown using PHYRE2 homology prediction. In green is shown the previously determined crystal structure for the bacterial rhomboid protease GlpG. In blue is the predicted structure for *SPCC790.03*, and the two structures are merged on the right to show their structural similarity. C) Schematic of Rbd2 protein, 251 aa. Catalytic residues are S130 and H182 (highlighted in red). Purple residues indicate missense point mutants obtained from a mutagenesis study. Black residues indicate the SHP box.

Figure 2.2: RBD2 promotes SREBP cleavage and requires rhomboid catalytic activity.

A) Wild-type and *rbd2Δ* or *sre1Δ* mutant yeast (200 cells) containing either a plasmid expressing Sre1N (Sre1 aa 1-440) or empty vector were grown on rich medium containing cobalt chloride ($CoCl_2$) or rich medium without $CoCl_2$. Sre1N, but not empty vector, rescued growth of the mutant strains on $CoCl_2$-containing medium, indicating that Sre1N was transcriptionally active and that the *rbd2Δ* mutant was unable to activate full-length Sre1. B) Western blot probed with antiserum to the Sre1 protein. Whole cell lysates were collected from wild-type and indicated mutant cells that were grown from 0-3.5 hours in the absence of oxygen. P and N denote the Sre1 precursor and nuclear forms, respectively. Asterisks denote background bands, as demonstrated by the *sre1D* stain in lane 13. The same blot, probed for actin, shows the relative protein loading across all 13

lanes. C) Wild-type and catalytically dead H182A *rbd2* genes were introduced into an *rbd2Δ* strain at the *his* locus under the control of the endogenous *rbd2* 5' and 3' UTRs. Whole cell lysates were prepared from cells grown in the presence or absence of oxygen, and probed with antiserum against Sre1. Cleavage was restored by wild-type (WT), but not H182A *rbd2*. D) Western blot of whole cell lysates from WT, *sre1Δ*, *rbd2Δ*, or *rbd2* missense mutant cells. Strains were grown in the presence or absence of oxygen to induce Sre1 cleavage. All missense mutations (lanes 5-10) failed to produce cleaved Sre1 protein, as indicated by antiserum to Sre1.

Figure 2.3: SREBPs are degraded by the proteasome in the rbd2Δ strain.

A) (*Experiment by Sumana Raychaudhuri*) *rbd2* was deleted in the *mts3-1* temperature-sensitive proteasome mutant background, and the resulting strain was grown at non-permissive temperature along with WT and *rbd2Δ* as controls. Whole cell lysates were probed for Sre2, and the *mts3-1*, *rbd2Δ* double mutant showed recovery of the precursor form of Sre2. B) A model substrate of Sre2 containing aa 423-793 with an N-terminal 3x FLAG epitope tag was expressed on a plasmid under control of a CaMV promoter in WT and *rbd2Δ* yeast. An empty vector control was included. Whole cell lysates were prepared from three transformants for each strain/plasmid combination, and western blots were probed with anti-FLAG M2 monoclonal antibody. The WT strain showed cleavage of the Sre2MS, while the *rbd2Δ* strain failed to cleave the Sre2MS. C) (*Experiment by Sumana Raychaudhuri*) The WT and *rbd2Δ* strains transformed with either Sre2MS or empty vector plasmid were treated with Bortezomib (Bz) at 1mM, and western blots of whole cell lysates were then probed with anti-FLAG M2 monoclonal antibody. The precursor form of the model substrate was recovered in the *rbd2Δ* strain upon treatment with Bz (lane 8).

Figure 2.4: SREBP degradation requires the Dsc E3 ligase complex.

A) Western blot for Sre2 showing Dsc-dependent degradation of Sre2 precursor protein. Double mutants were made with *dsc1Δ-dsc4Δ* and *rbd2Δ*. Whole cell lysates were probed for Sre2, and double mutants showed a recovery of Sre2 precursor protein. B) Western blot for Sre2 showing E3 ligase activity is required for Sre2 precursor degradation. A

double mutant of *rbd2Δ* and catalytically dead Dsc1 E3 ligase (*dsc1 C634A)* showed recovery of the Sre2 precursor protein.

Figure 2.5: Rbd2 and the Dsc complex localize to the Golgi, but do not physically interact.

A) Co-localization of Rbd2 with *cis*-Golgi marker Anp1 (top panel), late Golgi marker Sec72 (middle panel), and Dsc2 (bottom panel). A plasmid expressing Rbd2-6X-mCherry under the control of a CaMV promoter was transformed into strains for microscopy. All strains had an *rbd2Δ* background and either Anp1-GFP, Sec72-GFP, or *dsc2Δ* reconstituted with Dsc2-6X-GFP at the *his* locus. Strains were imaged on a confocal microscope, and individual sections were used to determine co-localization. Co-localization was determined by adjusting the minimum and maximum pixel intensities to reduce background in each individual channel (red or green), then generating a merged image with adjusted single-channel images. B) Co-immunoprecipitation of Dsc2 with TAP-tagged Rbd2 and Dsc1. In 10X bound fractions, Dsc1-TAP co-purified Dsc2 (lane 8), while Rbd2-TAP failed to do so (lane 9). Dsc2 signal was specific to co-immunoprecipitation as shown by a WT (no TAP) control (lane 7).

Figure 2.6: Rbd2 interacts with Cdc48 through its C-terminal SHP box to promote SREBP cleavage.

A) Alignment of human p47, human Ufd1, and *S. cerevisae* Dfm1 SHP box sequences with Rbd2 SHP box sequence. An asterisk indicates conserved residues. B) GST pull-down assay showing Rbd2-Cdc48 interaction. GST-fused Rbd2 C-terminus (aa 200-251), Dsc5 UBX (aa 251-247), Dsc2 UBA (aa 298-372), and GST-HA-V5 control were bound to MagneGST beads. Unbound proteins were removed and beads were washed before addition of *S. pombe* cytosol from KGY425 WT cells. Bound fraction was probed for Cdc48 and Ub. Rbd2 C-terminus bound Cdc48, but not Ub. Rbd2-Cdc48 interaction was comparable to Dsc5 UBX-Cdc48. C) Truncated Rbd2 C-terminus shows reduced binding to Cdc48. Assay was performed as in (B), but constructs were used with truncated forms of Rbd2 C-terminus. Amino acids included in the constructs are listed under GST-bait, and were comprised of four constructs: aa 200-251 (full-length C-terminus), aa 200-225

(N-terminal half), aa 225-251 (C-terminal half), and aa 200-240 (SHP delete). A construct deleting the second half of the C-terminus (aa 200-225, lane 3) completely failed to bind Cdc48, while a construct deleting the last 11 amino acids (aa 200-240, lane 5), which contained the predicted SHP box, had strongly reduced binding to Cdc48 when compared with the full-length construct. D) (*Experiment by He Gu*) Point mutations in the Rbd2 SHP box disrupt Cdc48 binding. Individual mutation of either of the conserved glycine residues (G244 and G246) abolished binding to Cdc48 in the *in vitro* binding assay (performed as in (B)). E) Deletion of SHP box reduces Sre1 cleavage. Strains were generated where the C-terminus of Rbd2 was truncated and replaced with a TAP tag. Two truncation mutants were made, a ΔSHP mutant (missing aa 240-251), and a Δ26 mutant (missing aa 225-251). Mutant cells and WT, *rbd2Δ*, and Rbd2-TAP control strain were grown in the presence or absence of oxygen, and whole cell lysates were collected. A western blot of the whole cell lysates showed the ΔSHP mutant had an Sre1 cleavage defect compared to the WT and Rbd2-TAP controls. The cleavage defect was not complete, however, as some nuclear form was still produced. The Δ26 mutant also had an Sre1 cleavage defect, and phenocopied the *rbd2Δ* strain, but probing the same whole cell lysates for TAP (F) showed that this mutant had low expression of the Rbd2 protein. F) Western blotting for TAP was performed on whole cell lysates from WT, Rbd2-TAP, and the ΔSHP and Δ26 truncation mutants. The ΔSHP mutant had a slightly reduced level of Rbd2 protein compared to Rbd2-TAP full-length, and the Δ26 mutant had an almost undetectable level of Rbd2 protein.

Figure 2.7: Model of SREBP cleavage in S. pombe

A) This study led me to hypothesize that the Dsc E3 ligase complex is "marking" the SREBPs with ubiquitin prior to the action of Rbd2. Cdc48 may be facilitating the recruitment of Rbd2 to the Dsc complex and Ub-SREBP substrates. Rbd2 acts to facilitate cleavage, and the SREBP substrates are then further processed to generate the active transcription factor product.

Supplemental Figure 2.1: Putative Arf/GEF from GI study not defective in Sre1 cleavage

A) Deletion of the gene *SPAC11e.11c* does not cause a defect in Sre1 cleavage. Western blots of whole cell lysates from WT, *dsc1Δ*, and *SPAC11e.11cΔ* strain was probed for Sre1 and Sre2. WT showed cleavage for both SREBP proteins, while *dsc1Δ* showed a cleavage defect for both SREBPs. The *SPAC11e.11cΔ* strain showed no cleavage defect for Sre1, but showed a cleavage defect for Sre2. B) The Sre2MS was transformed into WT, *dsc1Δ*, and *SPAC11e.11cΔ* strains, and a western blot of whole cell lysates was probed for FLAG. Cleavage of the Sre2MS followed the pattern of Sre1 as described in (A); cleavage occurred in WT and *SPAC11e.11cΔ*, but not in the *dsc1Δ*.

Supplemental Figure 2.2: Epistasis analysis of cdc48 and ubc4 with rbd2 shows UPS components act upstream of rhomboid protease

A) Epistasis analysis of *rbd2* and *cdc48*. Western blot of whole cell lysates probed for Sre1. Strains were grown from 0-4 h in $-O_2$ conditions. Wild-type strain showed increased cleavage at 2 and 4 h, *rbd2Δ* strain had reduced levels of Sre1 precursor at all time points without any cleavage, and *cdc48 E325K* strain showed a cleavage defect at all time points. The *rbd2Δ cdc48 E325K* double mutant showed an identical phenotype to the *cdc48 E325K* single mutant, indicating that it acts upstream of *rbd2*. B) Epistasis analysis of *ubc4* and *rbd2*. Western blot of whole cell lysates probed for Sre2. Strains were grown at either permissive temperature (25°C) or non-permissive temperature (36°C) for two hours before lysates were prepared using NaOH lysis method. Wild-type cells cleaved Sre2 and both 25°C and 36°C, *rbd2Δ* cells had an absence of Sre2 protein, and *ubc4 ts*$^-$ strain had a slight cleavage defect at 36°C. The *rbd2Δ ubc4 ts*$^-$ strain had a cleavage defect both at 25°C and 36°C, but the Sre2 did not disappear as in the *rbd2Δ* single mutant, indicating that the UPS likely acts upstream of *rbd2*.

Tables

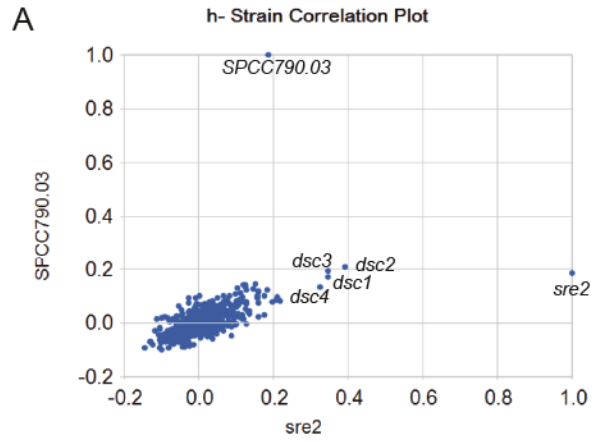
Table 2.1: *rbd2* alleles isolated from genetic selection

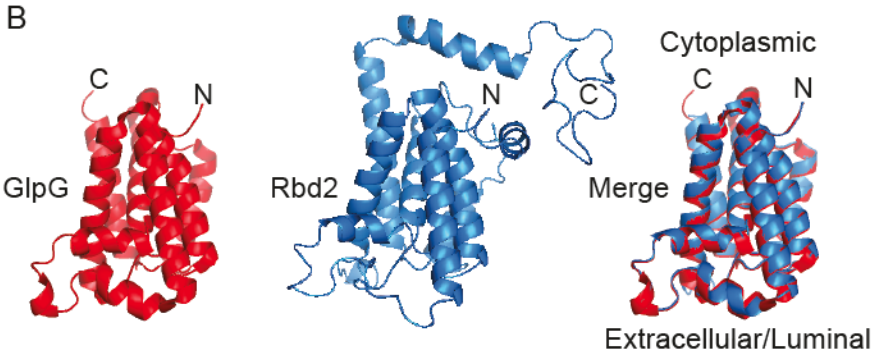
Allele Name	Mutation
rbd2-1	A127D
rbd2-2	A186T
rbd2-3	G128V
rbd2-4	G128V
rbd2-5	G128V
rbd2-6	E11*

Table 2.2: strains used in this study

Strain	Genotype	Source
KGY425	Wild-type	ATCC
YCN17	*dsc1Δ*	Stewart 2011
ESY180	*dsc2Δ*	Stewart 2011
ESY177	*dsc3Δ*	Stewart 2011
ESY183	*dsc4Δ*	Stewart 2011
YCN85	*dsc5Δ*	Stewart 2012
DJY121	*rbd2Δ*	This study
DJY304	*rbd2Δ anp1-GFP*	This study
DJY454	*rbd2Δ dsc2Δ dsc2-6X-GFP*	This study
PEY874	*mts3-1*	Gordon 2008
DJY450	*rbd2Δ sec72-GFP*	This study
DJY153	*rbd2Δ dsc1Δ*	This study
DJY163	*rbd2Δ dsc2Δ*	This study
DJY165	*rbd2Δ dsc3Δ*	This study
DJY169	*rbd2Δ dsc4Δ*	This study
DJY171	*rbd2Δ dsc5Δ*	This study
ESY58-107	*rbd2 G128V*	This study
DJY103	*rbd2 A127D*	This study
DJY109	*rbd2 A186T*	This study
SRY180	*rbd2Δ mts3-1*	This study
DJY472	*rbd2Δ dsc1 C634A*	This study
DJY123	*rbd2-TAP*	This study
YCN57-1	*dsc1-TAP*	Stewart 2011
DJY430	*rbd2-ΔSHP-TAP*	This study
DJY426	*rbd2-Δ26-TAP*	This study
PEY993	*cdc48 E325K*	Frost, 2012
DJY424	*rbd2Δ cdc48 E325K*	This study
PEY522	*sre1Δ*	Hughes 2005
PEY553	*sre2Δ*	Hughes 2005

Figure 2.1


A
h- Strain Correlation Plot
SPCC790.03
dsc3
dsc2
dsc1
dsc4
sre2
SPCC790.03
sre2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.2
0.0
0.2
0.4
0.6
0.8
1.0


B
C
N
GlpG
N
C
Rbd2
Cytoplasmic
C
N
Merge
Extracellular/Luminal

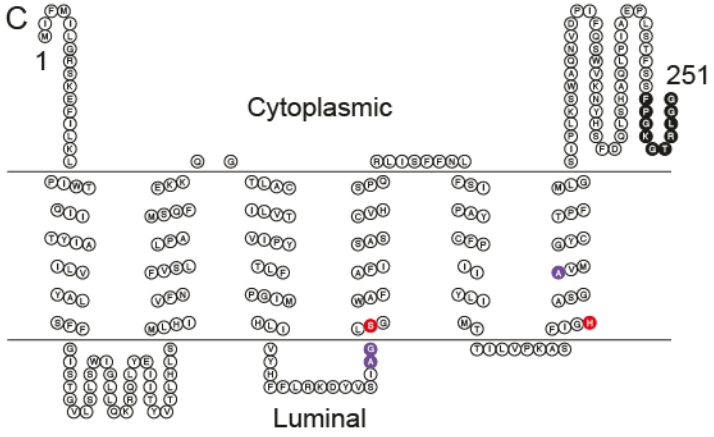

C
1
Cytoplasmic
251
Luminal

Figure 2.2

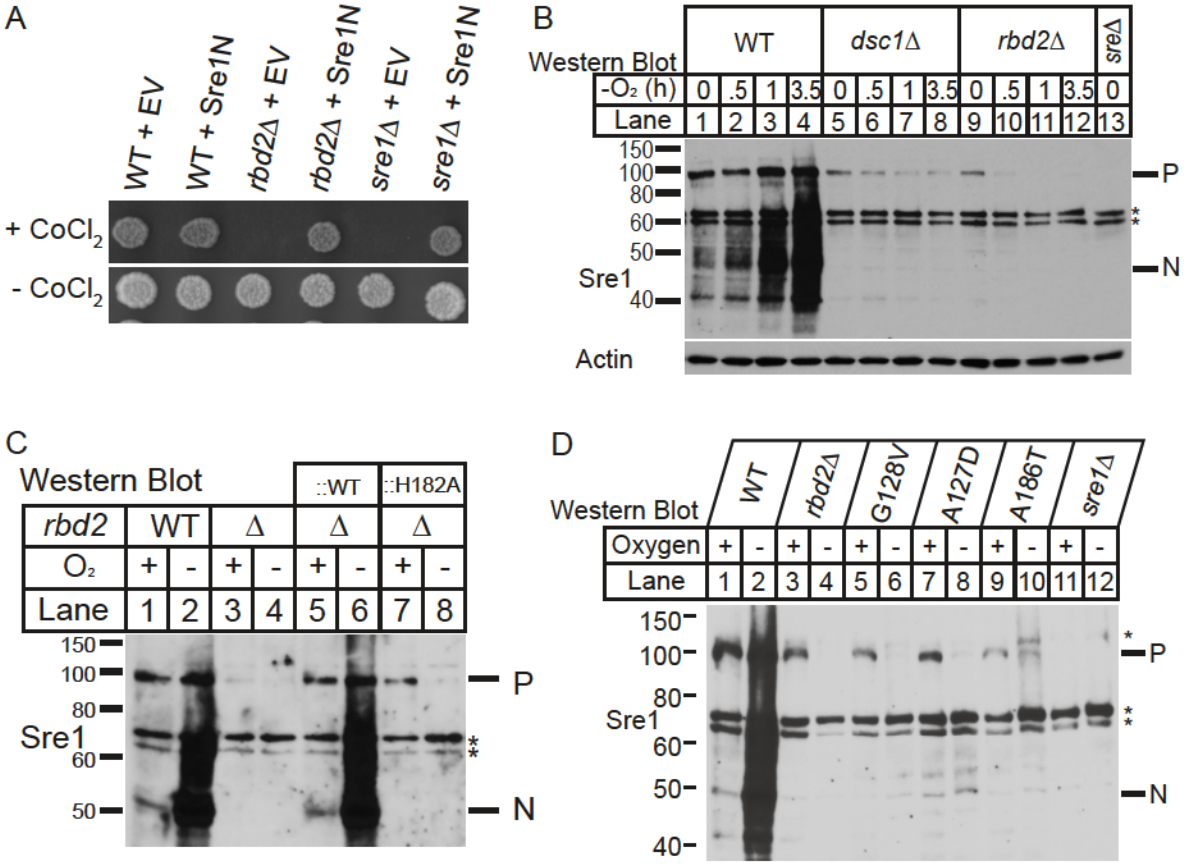

A
WT + EV
WT + Sre1N
rbd2Δ + EV
rbd2Δ + Sre1N
sre1Δ + EV
sre1Δ + Sre1N
+ CoCl2
- CoCl2
B
Western Blot
WT
dsc1Δ
rbd2Δ
sreΔ
-O2 (h)
Lane
Sre1
Actin
P
N
C
Western Blot
::WT
::H182A
rbd2
WT
Δ
O2
Lane
Sre1
P
N
D
Western Blot
WT
rbd2Δ
G128V
A127D
A186T
sre1Δ
Oxygen
Lane
Sre1
P
N

Figure 2.3

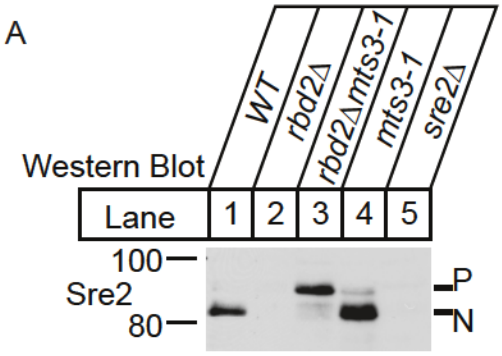
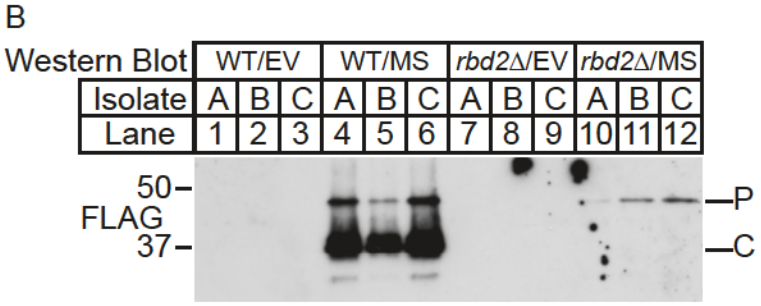


Figure by Sumana Raychaudhuri



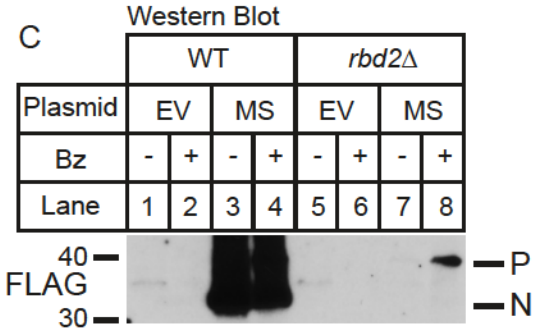


Figure by Sumana Raychaudhuri

Figure 2.4

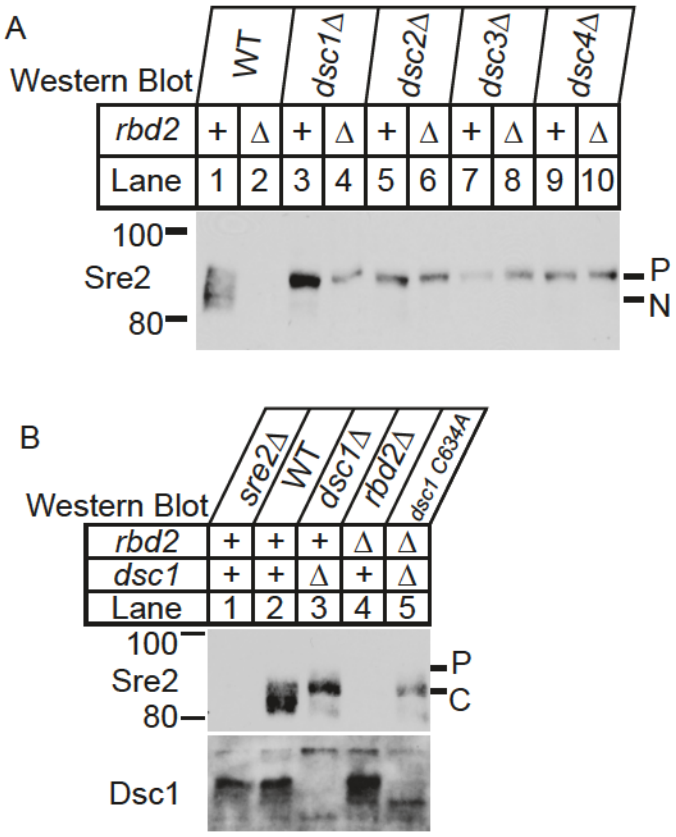

A
Western Blot
WT
dsc1Δ
dsc2Δ
dsc3Δ
dsc4Δ
rbd2
+ Δ + Δ + Δ + Δ + Δ
Lane
1 2 3 4 5 6 7 8 9 10
100
Sre2
80
P
N
B
Western Blot
sre2Δ
WT
dsc1Δ
rbd2Δ
dsc1 C634A
rbd2
+ + + Δ Δ
dsc1
+ + Δ + Δ
Lane
1 2 3 4 5
100
Sre2
80
P
C
Dsc1

Figure 2.5

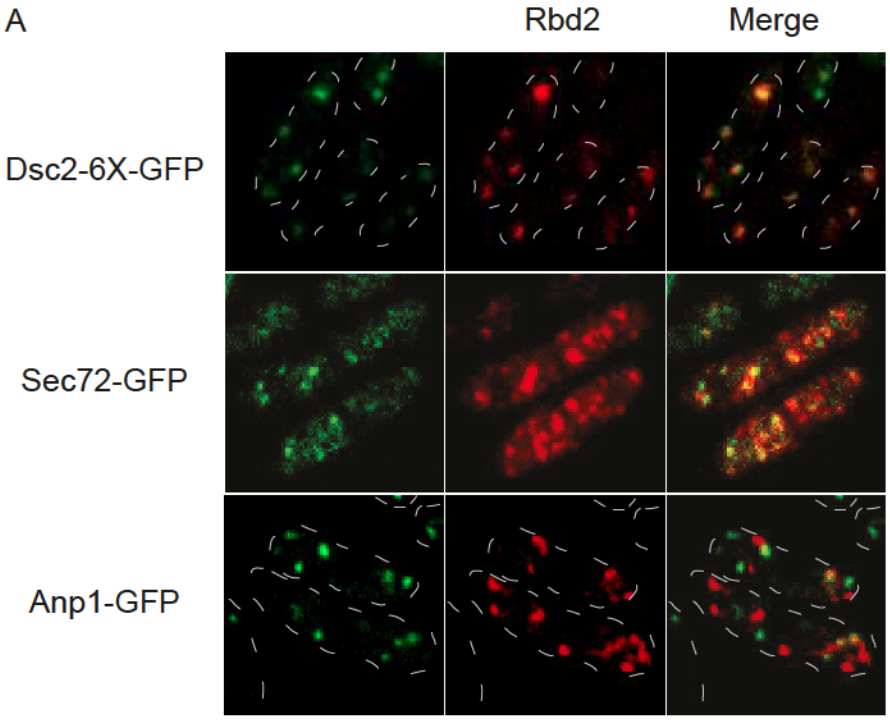

A
Rbd2
Merge
Dsc2-6X-GFP
Sec72-GFP
Anp1-GFP
B
IP: Anti-TAP
Input
Unbound
10x bound
Strain
WT
Dsc1T
Rbd2T
Lane
1
2
3
4
5
6
7
8
9
150
100
75
TAP
50
37
Dsc2
Dsc1-TAP
Rbd2-TAP

Figure 2.6

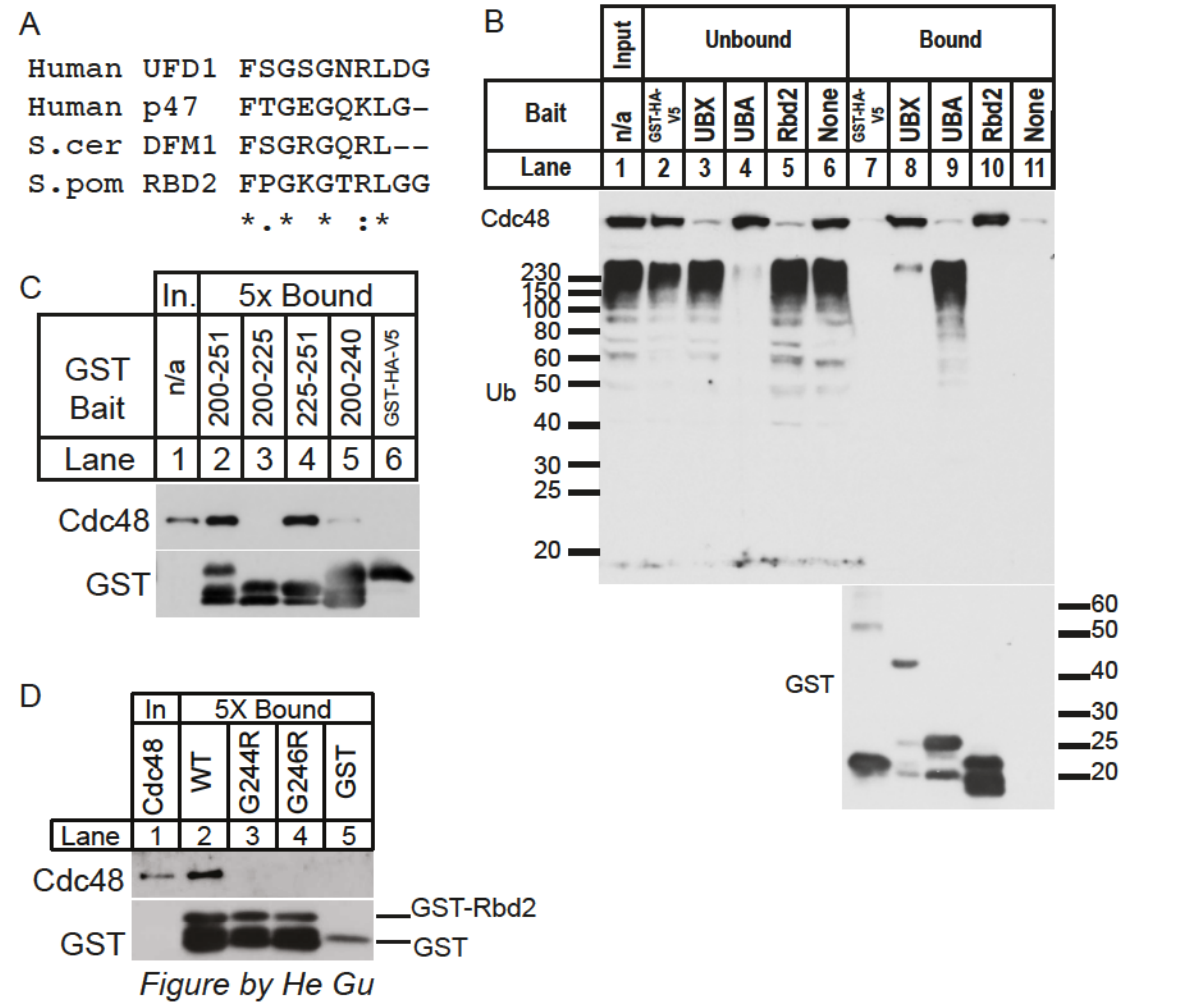
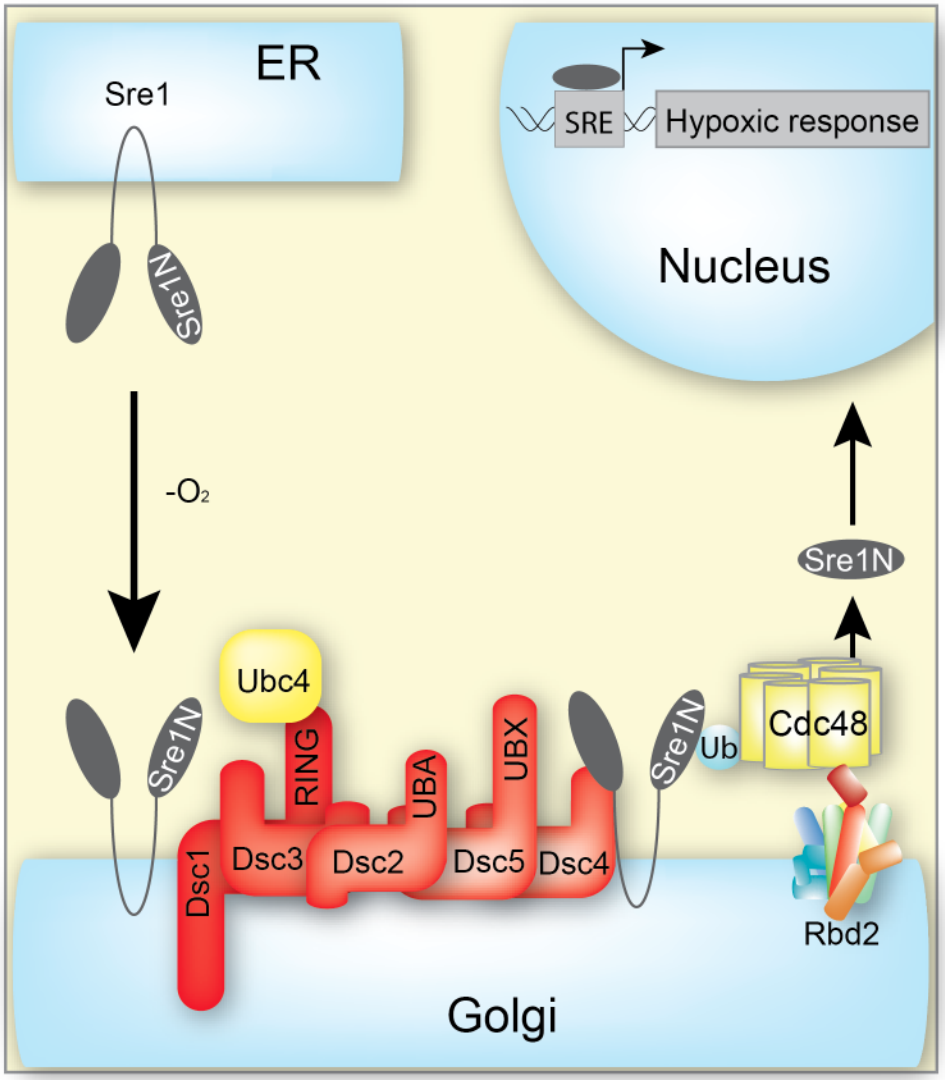

A
Human UFD1 FSGSGNRLDG
Human p47 FTGEGQKLG-
S.cer DFM1 FSGRGQRL--
S.pom RBD2 FPGKGTRLGG
. * :*
B
Input
Unbound
Bound
Bait
n/a
GST-HA-V5
UBX
UBA
Rbd2
None
Lane
Cdc48
Ub
GST
C
In.
5x Bound
GST Bait
n/a
200-251
200-225
225-251
200-240
GST-HA-V5
Lane
Cdc48
GST
D
In
5X Bound
Cdc48
WT
G244R
G246R
GST
Lane
Cdc48
GST
GST-Rbd2
GST
Figure by He Gu
E Western Blot
WT
sre1Δ
rbd2Δ
Rbd2-TAP
ΔSHP-TAP
ΔSHP-TAP
Δ26-TAP
O2
Lane
Sre1
P
N
F
Rbd2
WT
WT
ΔSHP
Δ26
TAP Tag
Lane
TAP
Rbd2 TAP

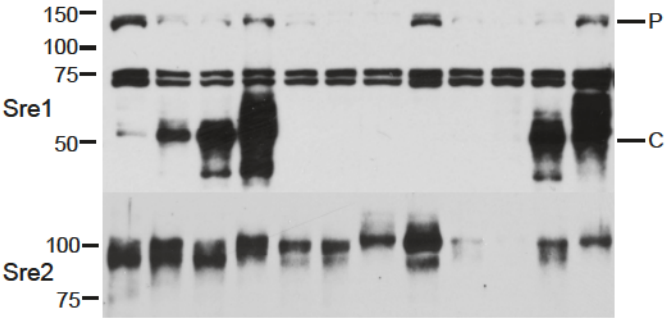
Figure 2.7


ER
Sre1
Sre1N
SRE
Hypoxic response
Nucleus
-O2
Sre1N
Ubc4
RING
UBA
UBX
Sre1N
Ub
Cdc48
Dsc1
Dsc3
Dsc2
Dsc5
Dsc4
Rbd2
Golgi

Supplemental Figure 2.1

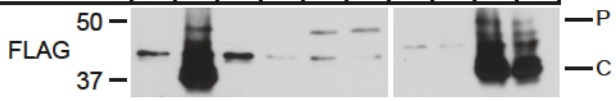
A

Western Blot	WT				*dsc1*Δ				*SPAC11E.11c*Δ			
Hours -O_2	0	1	3	6	0	1	3	6	0	1	3	6
Lane	1	2	3	4	5	6	7	8	9	10	11	12



B

Western Blot	WT		*dsc1*Δ				*SPAC11E.11c*Δ			
Hours -O_2	EV	MS	EV	EV	MS	MS	EV	EV	MS	MS
Lane	1	2	3	4	5	6	7	8	9	10

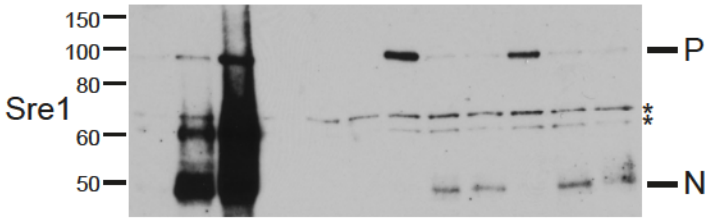


Supplemental Figure 2.2

A

Western Blot

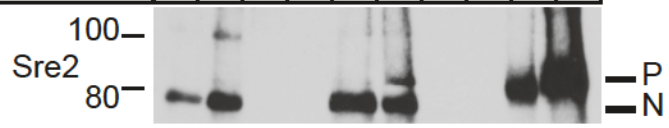
	WT			*rbd2Δ*			*cdc48 E325K*			*E325K rbd2Δ*		
-O_2 (h)	0	2	4	0	2	4	0	2	4	0	2	4
Lane	1	2	3	4	5	6	7	8	9	10	11	12



B

Western Blot

ubc4	WT		WT		*ts*		*ts*		*ts*	
rbd2	WT		Δ		WT		Δ		Δ	
Temp. (°C)	25	36	25	36	25	36	25	36	25	36
Lane	1	2	3	4	5	6	7	8	9	10



Chapter 3

The *Candida albicans* SREBP pathway

Abstract

Candida albicans, an obligate human commensal organism, colonizes the intestinal tract of its host. In immunocompromised individuals, *C. albicans* can cause opportunistic fungal infections originating from the commensal population. In other fungal pathogens such as *Cryptococcus neoformans* and *Aspergillus fumigatus*, sterol regulatory element binding protein (SREBP) is a key hypoxic transcription factor that has been shown to be required for host adaptation and virulence; in mammalian cells SREBP regulates lipid homeostasis. *C. albicans* has an SREBP homolog called Cph2, but known SREBP functions such as virulence, hypoxic response, and regulation of sterol homeostasis do not require *CPH2*. However, *C. albicans* host intestinal colonization requires *CPH2*, suggesting that this protein controls an undefined transcriptional program essential for commensalism. The regulatory mechanisms behind Cph2 cleavage and activation are also unknown. In mammalian cells and the basidiomycete *C. neoformans*, SREBP cleavage requires the Golgi-localized Site-1 and Site-2 proteases. Ascomycetous fungi lack a Site-2 protease homolog, and recent work in *Schizosaccharomyces pombe* and *A. fumigatus* identified a Golgi E3 ligase complex required for SREBP activation called the defective for SREBP cleavage (Dsc) E3 ligase. Here, I identify and begin to characterize the *C. albicans* Dsc E3 ligase, and show that *DSC3* is required for Cph2 cleavage.

Introduction

The obligate commensal organism *Candida albicans* colonizes the intestinal tract of its human host, and normally causes no disease (Shepherd 1985). However, immunocompromised individuals risk acquiring opportunistic infections originating from this commensal population of microflora (White 2007, Richardson 2005). Fungal pathogens account for 114-390 infections/million each year in the United States with *Candida spp.* constituting the largest number of infections and having high rates (30-40%) of attributable mortality (Pfaller 2010). *Candida albicans* accounts for the majority of these infections, making a clear understanding of the transition from commensal to pathogen a public health imperative. This transition suggests that distinct signals in the host environment induce different gene expression programs for colonization and pathogenesis. We are beginning to understand the pathogenic environment and genes required for infection, but less is known about the host intestinal environment and the adaptive responses necessary for productive colonization.

To date, research on *C. albicans* intestinal colonization has found that cells colonizing the mammalian intestinal tract have a gene expression profile distinct from cells grown in any laboratory-defined growth phase (Rosenbach 2010). These results indicate that unique growth conditions exist inside the host, but these have yet to be defined. Gene expression data suggest that the host intestinal environment has low oxygen and high iron and that cells induce expression of a specific set of *C. albicans* cell surface proteins (Rosenbach 2010, Chen 2011). It is unknown how the multiple host environment cues are integrated to yield successful intestinal colonization.

Kumamoto and colleagues identified *CPH2* in a search for genes upregulated during growth in the host intestine but not during laboratory-defined growth [11], and demonstrated that *cph2Δ/Δ* cells were defective for colonization of the mouse intestine. *CPH2* is a member of the sterol regulatory element binding protein (SREBP) family of transcription factors. It was first identified as a gene that promoted agar invasion of *S. cerevisiae* cells when heterologously expressed (Lane 2001, Rosenbach 2010), and codes for an 853 amino acid protein with SREBP membrane topology (Fig. 1) (Jones 2004). The N-terminal transcription factor domain binds the *TEC1* promoter at SRE sequences that resemble those for mammalian and fission yeast SREBP (Lane 2001). *C. albicans*

Cph2 is the least well-characterized SREBP among the principal opportunistic fungal pathogens.

In mammalian cells, SREBPs regulate lipid homeostasis (Shao 2012). Maintaining homeostasis is an essential part of cell growth and survival that requires the ability to respond to changing nutrient supplies. Mammalian SREBPs are membrane-bound, basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factors that regulate lipid homeostasis in response to cholesterol (Espenshade 2007, Goldstein 2006). SREBPs are synthesized in the endoplasmic reticulum and inserted as a hairpin with the transcription factor domain in the cytosol. When cells have sufficient cholesterol, SREBP is retained in the ER as an inactive precursor. When cells are cholesterol-deficient, SREBP is transported to the Golgi where it undergoes two sequential proteolytic cleavage events by the Site-1 and Site-2 proteases. The released N-terminus enters the nucleus to activate genes required for cholesterol synthesis and uptake, thereby restoring homeostasis. Studies in *S. pombe* revealed that SREBPs function as hypoxic transcription factors required for adaptation to oxygen-poor environments (Chang 2007, Todd 2006, Hughes 2005). The *S. pombe* SREBP, called Sre1, is activated by hypoxia and required for low oxygen growth (Hughes 2005). Under low oxygen, sterol synthesis is inhibited because it is oxygen-dependent, leading to proteolytic activation of Sre1. Gene expression studies showed that Sre1 upregulates enzymes in multiple pathways required for adaptation to low oxygen (Todd 2006).

To understand whether SREBP functions broadly as a hypoxic transcription factor, the function of the SREBP homolog was examined in the fungal pathogen *Cryptococcus neoformans*. These experiments were guided by the hypothesis that the host environment is hypoxic and that Sre1 is required for growth under these conditions. It was subsequently shown that *C. neoformans* Sre1 is a hypoxic transcription factor required for virulence in a mouse model of cryptococcosis, and gene expression studies show that *C. neoformans* Sre1 controls sterol synthesis (Chang 2007, Chun 2007). Further studies with *Aspergillus fumigatus* demonstrated that the SREBP ortholog SrbA is also a hypoxic transcription factor, essential for hypoxic growth, sterol homeostasis, and virulence in a mouse model of aspergillosis (Blatzer 2011, S. P. Willger 2008). Collectively, these data demonstrate that SREBPs have conserved roles in sterol

homeostasis, the hypoxic response, and virulence across fungi.

Unlike mammals and *C. neoformans*, ascomycetes like *S. pombe* lack the Site-2 protease that releases the N-terminal domain of SREBP from the membrane (Bien, Chang, et al. 2009, Bien and Espenshade 2010) . Stewart *et al*. discovered that the Golgi-localized Dsc E3 ligase complex is required for Sre1 cleavage in *S. pombe* (E. Stewart, C. Nwosu, et al. 2011, Stewart, Lloyd, et al. 2012), and these results were confirmed in *A. fumigatus* (S. C. Willger 2012). The Dsc E3 ligase complex has five integral membrane subunits named Dsc1-Dsc5. Sre1 interacts with the Dsc E3 ligase complex and is processed to release the N-terminus from the membrane through an unknown mechanism (E. Stewart, C. Nwosu, et al. 2011, Stewart, Lloyd, et al. 2012). In this way, Sre1 is converted from an inactive precursor to the active nuclear transcription factor under conditions of low oxygen.

Known SREBP functions such as virulence, the hypoxic response, and regulation of sterol homeostasis do not require *C. albicans CPH2*. However, *C. albicans* host intestinal colonization requires *CPH2*, suggesting that this protein controls an undefined transcriptional program essential for commensalism (Rosenbach 2010). The regulatory mechanisms behind Cph2 cleavage and activation are also unknown. *C. albicans* contains homologs of all subunits of the Dsc E3 ligase complex, so I asked whether the Dsc E3 ligase in this organism was required for the cleavage of Cph2. Studying the mechanistic relationship between the SREBP transcription factor Cph2 and the Dsc E3 ligase complex is a logical place to begin exploring the nature of the host-commensal interactions of *C. albicans* and the human intestinal environment. Knowing specific requirements for colonization will allow comparison between the commensal and the pathogenic environments, elucidating potential targets for therapeutics and treatment of *C. albicans* infections.

Results

Cph2 is not a hypoxic transcription factor

SREBPs have been shown to be required for adaptation to low oxygen in *S. pombe*, so our lab wanted to test whether Cph2 was required for growth under low oxygen. Prior to my joining the lab, several rotation students worked to address this question (S. Julie-Ann Lloyd, unpublished) . Wild-type and *cph2Δ/Δ* cells were grown in the absence of oxygen for 10 hours, and growth was measured every hour. Cells were grown in the presence of oxygen to compare growth rates of the wild-type and *cph2Δ/Δ* strains, and no growth difference was observed in the presence of oxygen (Figure 3.1 A). In the absence of oxygen a slight growth defect became apparent at later time points, but the standard deviation was large in these experiments, and therefore it was difficult to determine if the effect of $-O_2$ on growth was real.

To further examine whether Cph2 responded to low oxygen, whole cell lysates were prepared from the samples grown at 0, 2, and 4 h $-O_2$ to assay Cph2 cleavage (S. Julie-Ann Lloyd, unpublished). Western blotting for Cph2 showed that wild-type cells had cleaved Cph2 present at 0 h $-O_2$, and the level of cleaved Cph2 did not change at 2 or 4 h $-O_2$ (Figure 3.1 B). The level of cleaved Cph2 at all $-O_2$ time points was equivalent to the level at $+O_2$. Taken together, these data indicate that Cph2 is not a hypoxic transcription factor.

To ask whether Cph2 was cleaved by a S2P- or Dsc-dependent mechanism, I looked for homologs of the mammalian and fission yeast genes, respectively. I found that *C. albicans* did not contain a S2P homolog, but did contain homologs of *dsc1-dsc5* (Table 3.1). I selected one of these genes to delete and to ask if the function of the Dsc E3 ligase that was demonstrated in *S. pombe* was conserved in *C. albicans*. Because *C. albicans* has a diploid genome, homologous recombination must be used to delete both copies of a given gene. I selected *orf19.3517*, the *dsc3* homolog, to test whether a *dsc* deletion would yield a Cph2 cleavage defect. I deleted the first copy of *dsc3* using the auxotrophic marker strategy from Davis *et al*., and prepared whole cell lysates from the haploid deletion strains to determine if the *dsc3* gene was haploinsufficient. A *dsc3+/Δ* mutant did show a Cph2 cleavage defect compared to wild-type (Figure 3.2 A, lanes 1

and 3), although a second *dsc3+/Δ* isolate had a less severe Cph2 cleavage defect (Figure 3.2 A, lanes 1, 3, and 4). I deleted the second allele of *dsc3* in the *dsc3+/Δ* strain background using the drug selection strategy from Reuss *et al*. The *dsc3Δ/Δ* homozygous deletion strain failed to cleave Cph2 (Figure 3.2 B). This cleavage defect led to an accumulation of the precursor in the *dsc3Δ/Δ* strain, indicating that the Dsc E3 ligase was required for *C. albicans* SREBP cleavage.

Discussion

C. albicans Cph2 is a transcription factor of unknown function. It is predicted that Cph2 is an SREBP, based on the transmembrane topology and sequences predicting the SRE-binding capabilities. Taken with the previously published data by Lane et al. showing that Cph2 binds SRE-like sequences in the promoter of *TEC1* (Lane 2001), our lab was interested in whether Cph2 was required for adaptation to low oxygen. In growth assays performed by S. Julie-Ann Lloyd, low oxygen growth did not seem to require Cph2. This experiment should be repeated to verify the result, but we tentatively concluded that Cph2 was not required for hypoxic growth. In other organisms, SREBPs function to regulate gene transcription in response to low sterols and low oxygen, and in some pathogenic fungi SREBPs are required for virulence. However, in *C. albicans*, Cph2 is constitutively cleaved in the presence and absence of oxygen, as shown by S-Julie Ann Lloyd. This suggests that Cph2 does not respond to low oxygen, and the lack of a Scap homolog suggests it also does not respond to low sterols. Furthermore, Cph2 is not required for virulence, but is required for colonization of the host intestine (Rosenbach 2010). Additional studies are needed to determine the transcriptional program controlled by Cph2, and the mechanism by which it influences host intestinal colonization. Studies using RNA-seq on different populations of *C. albicans* (grown in host vs. culture, commensal vs. pathogenic) and comparing the transcript levels between WT and *cph2Δ/Δ* strains may begin to answer these questions.

Previously, no conditions were identified where Cph2 cleavage was inhibited. In the fission yeast *S. pombe* and in mammalian cells, SREBPs are cleaved in response to low oxygen and low sterols, respectively, and are held in the membrane-bound precursor form until stimulated to be cleaved. In this study, for the first time, I showed that making a homozygous *dsc3Δ/Δ* mutant strain could inhibit Cph2 cleavage. The fact that Cph2 cleavage can be inhibited without degradation of the precursor implies that a condition might exist where Cph2 cleavage is regulated, although experiments would be required to

test this hypothesis. Alternatively, Cph2 cleavage could be constitutive, and Cph2 could behave like *S. pombe* Sre2, which Cheung and Espenshade showed is always cleaved (Cheung 2013). Taken together with the fact that *C. albicans* does not have a Scap homolog, future studies should examine whether Cph2 behaves like Sre2. Current studies of the Dsc E3 ligase in *S. pombe* show that the complex regulates SREBP cleavage in response to low oxygen, but no additional conditions have been identified. Work in the budding yeast *Saccharomyces cerevisiae* has shown the presence of the Dsc E3 ligase in that organism (Ryan 2012), but *S. cerevisiae* lacks any SREBPs. Work in *C. albicans* may complement the studies in *S. cerevisiae*, and help identify additional conditions under which the Dsc E3 ligase is active.

The new knowledge that the Dsc E3 ligase regulates the cleavage of Cph2 yields a possible drug target for antifungal therapies. Further studies are required to determine how cleavage of Cph2 affects the activity of this transcription factor, and to explore the relationship between cleavage and intestinal colonization. Effective treatment of *Candida spp.* infections represents a major unmet clinical need. *Candida albicans*, as both an obligate commensal and pathogen, has an intricate and poorly understood relationship with its host environment. Several studies have examined the relationship between *C. albicans* and the host under pathogenic conditions (Mason 2012, White 2007, Nobile 2012, Karkowski-Kuleta 2009), but few have focused on the conditions important for non-pathogenic colonization (White 2007, Rosenbach 2010, Chen 2011). Given that the commensal population is the most common source of *C. albicans* infection in the immunocompromised host (Miranda 2009, Kumamoto 2011), understanding the requirements for host intestinal colonization is essential for the development of effective therapy.

Materials and Methods

Materials and Growth Media

The anti-Cph2 antibody was generated against amino acids 1-180 (excluding the DNA binding domain) using established protocols at Covance, Inc. YPD media was used to grow *C. albicans* (2% Bacto Peptone, 1% yeast extract, and 2% dextrose per mL), supplemented with uridine (80 µg/mL) for *ura*$^-$ strains. Selection for the Arg$^+$ transformants was done on synthetic medium (0.67% yeast nitrogen base plus ammonium sulfate and without amino acids, 2% dextrose, and 80 µg of uridine per mL).

Strain Construction

Strains were derived from *C. albicans* BWP17 wild-type (Wilson, Davis and Mitchell 1999). Strains used in this study are listed in Table 3.2. The *dsc3Δ/Δ* strain was constructed using a combination of methods. The initial *dsc3+/Δ* haploid strain was generated using an auxotrophic marker strategy as described by Kullas *et al* (Kullas, Li and Davis 2004). Briefly, BWP17 was transformed with the *dsc3::ARG4* cassette, which was amplified in a PCR using primers oDJ009 and oDJ010 and the pRS-ArgΔSpe template (Wilson, Davis and Mitchell 1999). Primers used in this study are listed in Table 3.3; plasmids used in this study are listed in table 3.4. The PCR product was purified, and transformed into BWP17 using a lithium acetate transformation protocol. Correct integration was demonstrated by PCR using oDJ011 and oDJ012 primers and genomic DNA template. The *dsc3Δ/Δ* strain was generated using the *SAT1* flipper method (Reuss, et al. 2004). Briefly, *dsc3+/Δ* was transformed with the *dsc3::FLP-caSAT1-FLP* cassette, generated using the protocol as described by Reuss *et al*. Transformation was performed using an electroporation protocol, and transformants were selected on YPD+uri+Nat. Insertion was confirmed by PCR and Southern blot.

Growth Assay

Candida albicans cells were grown in YPD media for 0-10 h. The $+O_2$ condition was grown in a normal incubating shaker at 30 °C, and the $-O_2$ condition was grown in a Ruskinn Invivo2 400 hypoxic workstation (Biotrace, Inc.) at 30 °C. A 1 mL aliquot of cells was removed at each time point and the OD_{600} was measured.

Western blotting

Samples were processed using NaOH lysis as previously described for *S. pombe* (Cheung 2013, Hughes 2005, E. Stewart, C. Nwosu, et al. 2011). Briefly, cells growing in log phase were centrifuged to remove medium and washed with water, then flash-frozen as cell pellets. Cell pellets were then stored at -80°C until protein was harvested. A BCA Assay (Thermo Scientific) was performed, and an equal amount of protein from each sample was loaded on an SDS-PAGE gel. Gels were transferred to nitrocellulose, blocked using a solution of 5% milk-PBST, and probed with anti-Cph2 antibody (1:2000 in 5% milk-PBST). Anti-rabbit HRP-coupled secondary antibody was used, and an ECL chemiluminescent reagent (Pico ECL, Thermo Scientific) was used to detect Cph2-specific bands.

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Special thanks to Oyindamola Oladosu and S. Julie-Ann Lloyd, rotation students in the Espenshade lab, for completing the work in Figure 3.1 during their rotations, and for generating the anti-Cph2 antibody. Thank you to Dr. Janet Staab, Dr. Dana Davis, and Dr. Brendan Cormack for reagents. Thank you also to Dr. Dana Davis for troubleshooting and discussion.

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Figure Legends

Figure 3.1: Cph2 is not a hypoxic transcription factor

A) *C. albicans* growth curve comparing wild-type and *cph2Δ/Δ* cells grown in the presence or absence of oxygen. Cells were grown in YPD media for 0-9 h, and the $O.D._{600}$ was measured at each hour. Both wild-type and *cph2Δ/Δ* cells grew more slowly in the absence of oxygen, but a difference between the two strains was difficult to determine, due to the large standard deviation at the later time points. B) Western blot comparing Cph2 levels in the presence and absence of oxygen. Wild-type and *cph2Δ/Δ* cells were grown in the presence or absence of oxygen for 0, 2, or 4 h. Whole-cell lysates were harvested, and a western blot of these samples was probed for Cph2. The western blot indicated that wild-type cells had a consistent amount of Cph2, and that all the Cph2 was in the cleaved form, demonstrating that Cph2 cleavage was not altered in response to hypoxia.

Figure 3.2: The putative Dsc E3 ligase facilitates Cph2 cleavage

A) Western blot showing the haploinsuffincicy of *DSC3*. Wild-type, *cph2Δ/Δ*, and two heterozygous *dsc3+/Δ* strains were grown to log phase in the presence of oxygen. Cells were harvested, and whole cell lysate was prepared. Western blotting on whole cell lysates for Cph2 showed that the two heterozygous *dsc3+/Δ* strains had a reduced amount of Cph2 cleavage compared to wild-type, but were still able to generate cleaved Cph2. Isolate DJY002 had a more severe cleavage defect compared to isolate DJY005, possibly reflecting a difference in the deleted allele. B) Western blot showing that a *dsc3Δ/Δ* homozygous deletion strain is unable to cleave Cph2. Experiment was performed as in (A) using wild-type, *cph2Δ/Δ*, and *dsc3Δ/Δ* strains. The *dsc3Δ/Δ* strain was unable to generate any cleaved Cph2, although the amount of precursor did not equal the amount of P+C in wild-type, suggesting possible regulation or positive feedback similar to the *S. pombe* system.

Tables

Table 3.1: Fungal SREBP Pathway Homologs

S. pombe	*C. albicans*
sre1	*CPH2/orf19.1187*
dsc1	*orf19.2131*
dsc2	*orf19.5984*
dsc3	*orf19.3517*
dsc4	*orf19.3565*
dsc5	*orf19.1595*

Table 3.2: Strains Used in This Study

Strain	Genotype	Source
BWP17	Wild-type	Wilson 1999
DJY002	*dsc3+/Δ*	This study
DJY005	*dsc3+/Δ*	This study
DJY050	*dsc3Δ/Δ*	This study
CBY111-1	*cph2Δ/Δ*	Lane 2001

Table 3.3: Primers Used in This Study

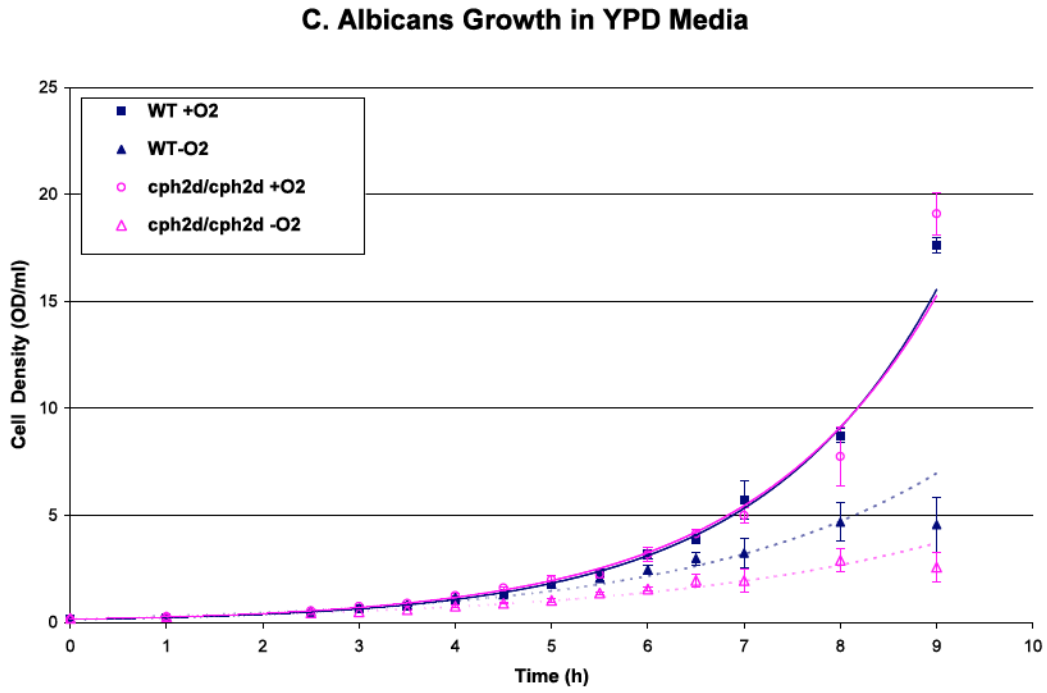
Oligo Number	Name	Sequence
oDJ009	*DSC3* 5 DR	AGCACATTAAATCCAATTAACATCCATTTTAAAACACCAAGAATT AAACTTCATCTATCATTTCCCAGTCACGACGTT
oDJ010	*DSC3* 3 DR	TACATTTGATTCAATGAGCACCAGTTTTATGTATATTTATATTGTA TTATATTGTAACTAGTGGAATTGTGAGCGGATA
oDJ011	*DSC3* 5 detect	GGCTCCTTTCACTTTGGAG
oDJ012	*DSC3* 3 detect	CAACCGGTCATCTTAGTGCCC
oDJ029	*DSC3* A – SAT flipper	CGGGGTACCTTAAACCAGGCTCGTATCTTCC
oDJ030	*DSC3* B – SAT flipper	CCGCTCGAGTGATAGATGAAGTTTAATTCTTGG
oDJ031	*DSC3* C – SAT flipper	TCCCCGCGGGGTCTTCCGGCCCTCACTGAAAAC
oDJ032	*DSC3* D – SAT flipper	CGAGCTCGTAGAATTTATTGAATGAGGGG
oDJ047	*caSAT1* 5 detect	GGGCACTAAGCAGACAGCTCC
oDJ048	*caSAT1* 3 detect	CATATGTGAAGTGTGAAGGGGGGAG

Table 3.4: Plasmids Used in This Study

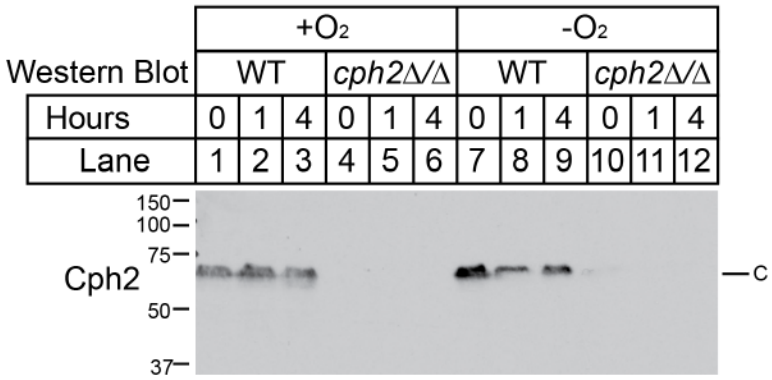
Plasmid Name	Gene; Marker	Source
pRS-ArgΔSpe	*ARG4*; n/a	Wilson 1999
pDJ01	Nourseothricin resistance	This study
pDJ02	Nourseothricin resistance	This study
pSFS1	Nourseothricin resistance	Reuss 2004

Figure 3.1

A


C. Albicans Growth in YPD Media
WT +O2
WT-O2
cph2d/cph2d +O2
cph2d/cph2d -O2
Cell Density (OD/ml)
Time (h)
0
5
10
15
20
25
0
1
2
3
4
5
6
7
8
9
10

B


+O2
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Western Blot
WT
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cph2Δ/Δ
Hours
0 1 4 0 1 4 0 1 4 0 1 4
Lane
1 2 3 4 5 6 7 8 9 10 11 12
150
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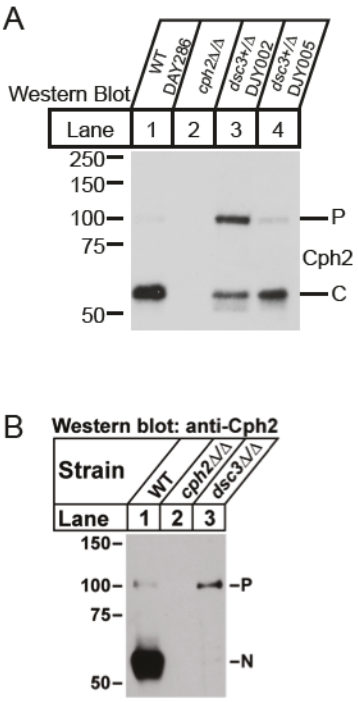
C

Figure 3.2


A
Western Blot
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cph2Δ/Δ
dsc3+/Δ DJY002
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Chapter 4

Conclusions

Thesis Summary

Herein I present my thesis work on the study of ascomycetous fungal SREBPs and their activation requiring the Dsc E3 ligase and Rbd2 rhomboid protease. In these studies, I (1) characterized the *S. pombe* ORF *SPCC790.03* and its gene product, the rhomboid protease Rbd2, (2) elucidated the relationship between *S. pombe* Rbd2 and the Dsc E3 ligase, and (3) began to characterize the *Candida albicans* Dsc E3 ligase and its relationship to Cph2.

Conclusions and Perspectives

SREBP Cleavage in Fission Yeast Requires the Rbd2 Rhomboid Protease

When I began this work, the Espenshade lab had recently identified the Dsc E3 ligase as being required for SREBP cleavage and activation in *S. pombe* (Stewart, Nwosu, et al. 2011, Stewart, Lloyd, et al. 2012). However, even with characterization of this complex, the model for SREBP cleavage was incomplete. The only proteolytic component implicated in cleavage was the proteasome, and experiments attempting to show a classic RUP mechanism for the proteasome-SREBP cleavage event were inconclusive. Additional studies were undertaken to look for genes involved in SREBP cleavage. A study using a genetic interaction (GI) mapping technique identified the uncharacterized ORF *SPCC790.03*, and I showed that the gene product of *SPCC790.03* is a rhomboid protease, called Rbd2, that is required for SREBP cleavage.

Initially, the idea of how a rhomboid protease might accomplish SREBP cleavage seemed relatively straightforward. The mammalian SREBP cleavage machinery uses an intramembrane protease (the Site-2 Protease, a Zn-metalloprotease) to catalyze the final cleavage event, so the identification of an intramembrane protease in the fission yeast system potentially meant that the two systems had more in common than previously thought. After characterizing Rbd2, I began to hypothesize that a combination of RIP and RUP mechanisms facilitated SREBP cleavage in fission yeast. Rbd2 is oriented in the membrane so that its N- and C-termini are both in the cytoplasm. This orientation places the catalytic site closer to the luminal face of the Golgi membrane, and based on the cleavage events that have been characterized for other rhomboid proteases, this indicated that Rbd2 was likely cleaving its substrates near the Golgi lumen. Close examination of the fission yeast SREBP, Sre2, by Cheung and Espenshade demonstrated that many of the residues in the second transmembrane segment were required for optimal cleavage of the protein; conversely, not many residues in the first transmembrane segment were required for cleavage (Cheung and Espenshade 2013).

Combining what is known about cleavage motifs in rhomboid substrates with the information from Cheung's study of Sre2, some speculation can be made as to where

Rbd2 may cleave Sre2. Rhomboid proteases prefer to cleave substrates that have a small residue in the P1 site (G, A, S, or C), and bulky, hydrophobic residues in the P4 and P2' sites (V, L, I, F, M, Y or W) (Strisovsky, Sharpe and Freeman 2009, Ha, Akiyama and Xue 2013). Using these criteria, I identified several putative cleavage sites in the area between the luminal loop and the end of the second transmembrane domain of Sre2 (Figure 4.1), indicating that it is possible that Sre2 is a direct substrate of Rbd2. However, if Sre2 is a substrate of Rbd2, it remains to be determined how Rbd2 processing contributes to the final Sre2N product, which was shown to contain only the cytoplasmic N-terminal portion of the protein (Cheung and Espenshade 2013). The genetic epistasis experiments I presented in Chapter 2 of this thesis indicate that the Dsc E3 ligase acts prior to Rbd2 to facilitate SREBP cleavage, but the results of the genetic epistasis experiments were less clear for Cdc48 and the UPS machinery. Further studies are needed to address whether these two genes are required before Rbd2 activity, after Rbd2 activity, or both before and after. Taken together, this evidence suggests a model where SREBPs are ubiquitinated by the Dsc E3 ligase, clipped by Rbd2 to separate the two transmembrane domains, and then further processed by the UPS machinery to generate the N-terminal transcription factors. This mechanism, if correct, represents the discovery of a novel, combination RIP/RUP cleavage mechanism.

Subsequent experiments to test this model will require isolating Rbd2 and Sre2 from the rest of the Dsc E3 ligase, likely via expression in a heterologous system or protein purification methods. Experiments can then be done to assess, directly, if Sre2 is a substrate of Rbd2. Many rhomboid proteases and their substrates have been analyzed in a similar fashion, yielding information about how the protease and substrate interact, the structural and sequence requirements of the substrate that allow cleavage to occur, and where the protease cleaves the substrate(Ha, Akiyama and Xue 2013, Strisovsky, Sharpe and Freeman 2009). Preliminary experiments indicate that Rbd2 and the Sre2MS can be co-expressed in mammalian cells, and that Rbd2 can cleave a bacterial protein, TatA, so further exploration will certainly inform the model of how Rbd2 participates in SREBP cleavage in fission yeast.

The Dsc E3 Ligase is Conserved in Candida albicans

After the Espenshade lab showed that the *Cryptococcus neoformans* SREBP, Sre1, was required for virulence, the field of fungal pathogenesis became interested in SREBPs in other opportunistic fungal pathogens; the Cramer lab found that *Aspergillus fumigatus* also had an SREBP required for virulence (SrbA). One main difference between *C. neoformans* and *A. fumigatus* is that *C. neoformans* has an S2P homolog, and therefore activates its SREBP using the RIP mechanism catalyzed by S2P (Chang, et al. 2007, Bien and Espenshade 2010). *A. fumigatus* has the Dsc E3 ligase complex, and studies showed that the components of the Dsc E3 ligase were required for activation of SrbA and therefore also required for virulence (Willger, Cornish, et al. 2012, Willger, Puttikamonkul, et al. 2008). Based on this emerging interest in the SREBP transcription factors in fungal pathogens, Cheung and Espenshade conducted a bioinformatic survey of fungi to determine which organisms contained SREBPs, S2P homologs, and Dsc E3 ligase homologs. They found that many fungi contained an SREBP, but that only basidiomycetes (including *C. neoformans*) contained a S2P homolog. This raised the question of whether or not the Dsc E3 ligase was widely conserved among ascomycetes. There have been three characterized examples of the Dsc E3 ligase in this group of fungi: *S. pombe*, *A. fumigatus*, and *Saccharomyces cerevisiae* (Stewart, Lloyd, et al. 2012, Stewart, Nwosu, et al. 2011, Ryan, et al. 2012, Willger, Cornish, et al. 2012). Interestingly, *S. cerevisiae* does not have an SREBP, a point that will be discussed in more detail below.

Preliminary studies in the Espenshade lab indicated that *Candida albicans* might be an interesting fungal pathogen in which to study SREBPs, because this organism had an SREBP, called Cph2, which did not appear to be required for virulence. Cph2 was shown to be important in colonization of the host intestinal tract, and our lab undertook studies to ask whether Cph2 was required for adaptation to low oxygen like *S. pombe* Sre1. Cph2 was not required for growth under low oxygen, and in the absence of any information about the regulation of Cph2, I decided instead to focus on how this transcription factor was activated. I showed that *C. albicans* contained homologs of the Dsc E3 ligase, and that *DSC3* was required for cleavage of Cph2. Logical next steps will be to examine whether Cph2 is active in its full-length precursor form (unlikely based on what is known about other SREBPS), or its cleaved form. It should also be examined

where Cph2 localizes in the cell, and what sort of transcriptional program is controlled by its activity. There is no known condition where Cph2 is not cleaved, so it will also be of interest to see whether or not cleavage is regulated. The possibility exists that Cph2 behaves similarly to *S. pombe* Sre2, so a connection to cell wall formation should be investigated.

One important implication from this work is that the Dsc E3 ligase may be a good drug target to treat opportunistic fungal pathogens such as *A. fumigatus* and *C. albicans*. Since *A. fumigatus* requires the activity of the Dsc E3 ligase for virulence and *C. albicans* likely requires the Dsc E3 ligase for colonization of the host intestinal tract, it follows that inhibition or disruption of this complex could be helpful in treating infections of these fungal species. Although Cph2 is not directly required for virulence in *C. albicans*, infections of this opportunistic pathogen frequently arise from the commensal population, so regulating the Dsc E3 ligase provides a potential therapeutic target for controlling commensalization of the host intestinal tract by *C. albicans* (Miranda, et al. 2009). An additionally promising fact is that mammals do not appear to have the Dsc E3 ligase. The closest mammalian homologs of the Dsc proteins are those in the gp78 E3 ligase, which is involved in ER-associated degradation (ERAD), however, the conservation is loose, and not all components are conserved (Lloyd, Raychaudhuri and Espenshade 2013). As mentioned in the introduction in Chapter 1 of this thesis, a common interest in the field of fungal pathogenesis is identifying physiological differences between fungi and mammals, and then exploiting these differences as drug targets for the treatment of fungal infections. Targeting the Dsc E3 ligase, rather than the SREBPs, for the treatment of fungal infections is more logical, because although mammals use SREBPs, there has not been an identified complex homologous to the Dsc E3 ligase in mammals.

Protein Quality Control: A Conserved Role for Rhomboid Proteases?

One of the most exciting implications that emerged from my thesis work is the possibility that rhomboid proteases have a conserved function in protein quality control. A study by Fleig *et al.* in 2012 concluded that the mammalian rhomboid protease, RHBDL4, played a role in ERAD. They showed that RHBDL4 had an extended C-terminus that was capable of binding both ubiquitin and p97 (the mammalian homolog of

Cdc48). In *S. pombe*, the Dsc E3 ligase was proposed to have quality control functions in the Golgi distinct from the known functions of other E3 ligase complexes in ERAD (Lloyd, Raychaudhuri and Espenshade 2013). Identifying Rbd2, a rhomboid protease, lent further support to the Golgi quality control model based on the work by Fleig *et al*. The parallels between RHBDL4 and Rbd2 were immediately striking to me, because Rbd2 also has the ability to bind Cdc48 through its C-terminus.

RHBDL4 and Rbd2 are both in the secretase-B class of rhomboid proteases, and RHBDL4 is the closest mammalian homolog of Rbd2, structurally. The fact that structural conservation may be mirrored in function was intriguing, and I decided to look at other fungal rhomboid proteases to see if they retained the extended C-terminus structure that contained the Cdc48-binding motif in RHBDL4 and Rbd2. I aligned the sequences of several fungal and human rhomboids, and found that the fungal Rbd2 rhomboid proteases clustered with RHBDL4 (Figure 4.2 A). I then used Phobius topology prediction (Kall, Krogh and Sonnhammer 2004) to determine the extent of the C-termini for the rhomboids in the alignment (Table 4.1), and repeated the multiple sequence alignment using only the sequences from the extended C-terminal regions (Figure 4.2 B). The alignment suggested that other fungal rhomboids, such as *S. cerevisiae* Rbd2, might also have an SHP-like sequence in their C-termini, and therefore retain the Cdc48 binding capability I characterized in *S. pombe* Rbd2. Preliminary experiments have been conducted to test whether the *S. cerevisiae* Rbd2 C-terminus can bind to Cdc48 *in vitro*, and results suggest that this is indeed the case (He Gu, unpublished data). Taken together with the fact that *S. cerevisiae* has the Dsc E3 ligase complex, but no SREBPs, these data all support a model where the Dsc E3 ligase works with Rbd2 to accomplish Golgi protein quality control. Further experiments are needed to characterize *S. cerevisiae* Rbd2 and to identify possible quality control substrates of the Dsc E3 ligase and Rbd2 in this organism.

Final Conclusions

The work I presented in this thesis answered some of the open questions in the fields of regulated proteolysis by RIP and RUP, rhomboid protease functions, and the role of the Dsc E3 ligase complex in ascomycetous fungi. As these experiments show, the *S. pombe* Rbd2 rhomboid protease clearly has a role in SREBP cleavage, possibly by cleaving the SREBP substrates directly. They also show that the Dsc E3 ligase has a conserved function of SREBP cleavage in *C. albicans*. These experiments pave the way for an exploration of the cleavage mechanism in *S. pombe*, Golgi protein quality control, and conservation of these features among other fungal species such as *S. cerevisiae* and *C. albicans*.

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Figure Legends

Figure 4.1:

A) Sre2 Model Substrate (Sre2MS) cleavage requirements, adapted from Cheung 2013. Residues that were mutated in the study are indicated by squares, and a red color indicates a strong block in cleavage (precursor > cleaved); gray indicates a partial block in cleavage (precursor = cleaved). B) Potential rhomboid cleavage sites in Sre2. The area from the end of the first transmembrane to the end of the second transmembrane domain was analyzed for the presence of potential rhomboid cleavage sites. The possible P1 sites were highlighted in blue, and used to determine what the P4 (green) and P2' (purple) residues would be based on that particular P1 site. The cleaved bond is indicated with a dash. Combining this analysis from the information from figure (A), option (b) looks promising, due to the requirement for the P4 lysine in Sre2MS cleavage, the isoleucine in the P2' position, and the location in the luminal juxtamembrane region.

Figure 4.2: Multiple sequence alignments of eukaryotic rhomboids indicate conserved C-terminal Cdc48 binding

A) Alignment tree made from multiple sequence alignment of eukaryotic rhomboid proteases. The human rhomboid protease RHBDL4 clustered more closely with the fungal Rbd2 homologs, and was closest to *S. cerevisiae* Rbd2. B) Multiple sequence alignment of C-termini from rhomboid proteases. Based on the information in Table 4.1, the eukaryotic rhomboid proteases that had C-termini were aligned using only the C-terminal sequences. Alignment quality scores increased in the area containing the Rbd2 SHP box, indicating that other eukaryotic rhomboids may also contain this Cdc48 binding motif.

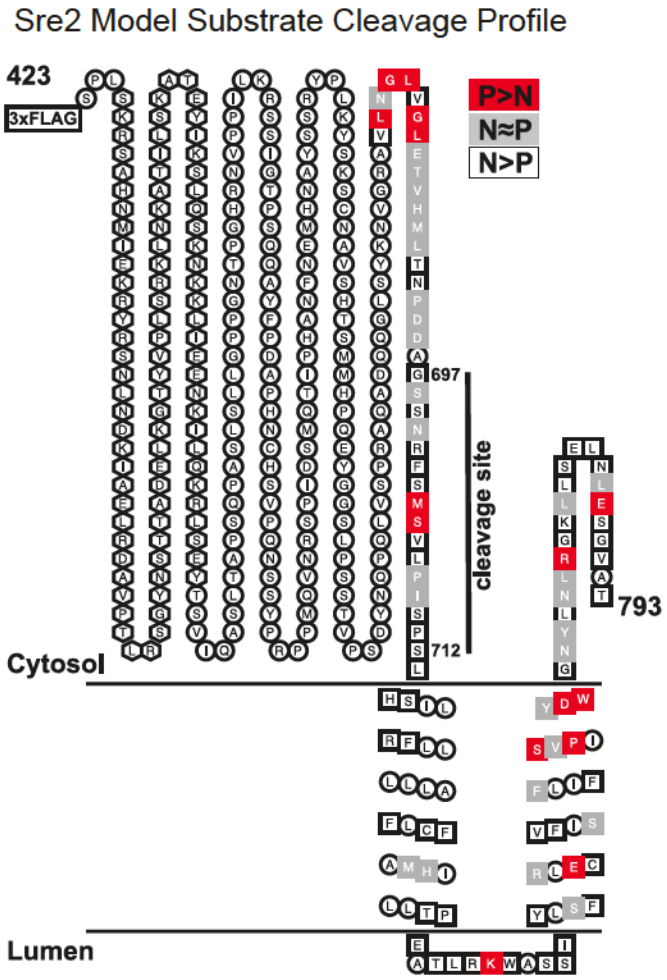
Tables

Table 4.1: Analysis of rhomboid C-termini

Rhomboid Name	**Species**	**Extended C-terminus? (length)**
RHBDL1	Human	No
RHBDL2	Human	No
RHBDL3	Human	No
RHBDL4	Human	Yes, aa 206-315 (109)
PARL	Human	Yes, aa 348-379 (31)
Sc Rbd2	*S. cerevisiae*	Yes, aa 190-262 (72)
Sp Rbd2	*S. pombe*	Yes, aa 197-251 (54)
Sj Rbd2	*S. japonicus*	Yes, aa 139-256 (117)
Ca Rbd2	*C. albicans*	Yes, aa 203-284 (81)
Cg Rbd2	*C. glabrata*	Yes, aa 213-266 (53)
Af Rbd2	*A. fumigatus*	Yes, aa 211-272 (61)

Figure 4.1

A

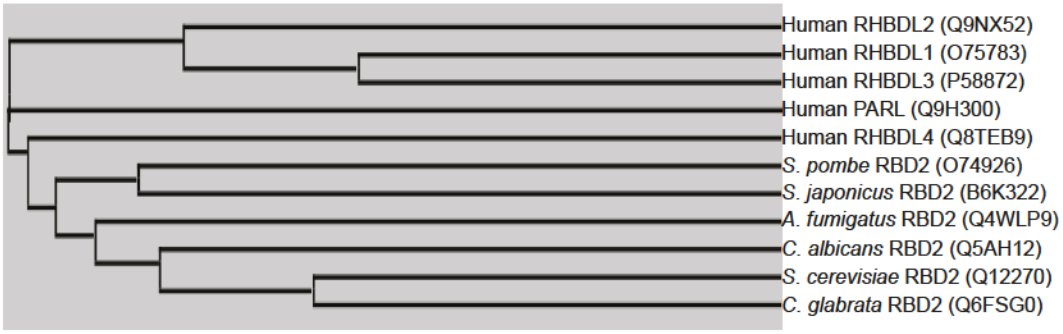


B

```
A. N- EATLRKWA-SSIYLSFRLECVFISFLIFSVPIYDWGNYL -C
B. N- EATLRKWAS-SIYLSFRLECVFISFLIFSVPIYDWGNYL -C
C. N- EATLRKWASS-IYLSFRLECVFISFLIFSVPIYDWGNYL -C
D. N- EATLRKWASSIYLS-FRLECVFISFLIFSVPIYDWGNYL -C
E. N- EATLRKWASSIYLSFRLEC-VFISFLIFSVPIYDWGNYL -C
F. N- EATLRKWASSIYLSFRLECVFIS-FLIFSVPIYDWGNYL -C
G. N- EATLRKWASSIYLSFRLECVFISFLIFS-VPIYDWGNYL -C
```

Figure 4.2

A


Human RHBDL2 (Q9NX52)
Human RHBDL1 (O75783)
Human RHBDL3 (P58872)
Human PARL (Q9H300)
Human RHBDL4 (Q8TEB9)
S. pombe RBD2 (O74926)
S. japonicus RBD2 (B6K322)
A. fumigatus RBD2 (Q4WLP9)
C. albicans RBD2 (Q5AH12)
S. cerevisiae RBD2 (Q12270)
C. glabrata RBD2 (Q6FSG0)

B


MSA
The multiple sequence alignment result as produced by T-coffee.
T-COFFEE, Version_9.03.r1318 (2012-07-12 19:05:45 - Revision 1318 - Build 366)
Cedric_Notredame
SCORE=35
BAD AVG GOOD
sp|Q8TEB9|RHBL4 : 27
sp|Q9H300|PARL : 21
sp|Q12270|RBD2 : 33
sp|Q5AH12|RBD2 : 35
tr|B6K322|B6K32 : 21
sp|Q4WLP9|RBD2 : 32
sp|Q6FSG0|RBD2 : 30
sp|O74926|RBD2 : 30
cons : 35

Appendix A

Localization of *Schizosaccharomyces pombe* Dsc5

Abstract

The *Schizosaccharomyces pombe* Dsc E3 ligase is a Golgi-localized protein complex that participates in SREBP cleavage. Preliminary experiments indicated that one of the members of the Dsc E3 ligase, Dsc5, exists independently in the cell, and is not always complexed with the other Dsc proteins. To ask where Dsc5 was localized, live-cell fluorescence microscopy was used to look at Dsc5-GFP. I found that Dsc5 was partially localized to the Golgi, as expected based on the localization of the other Dsc E3 ligase components; However, Dsc5 was also localized partially to the endoplasmic reticulum. This raises the question of whether Dsc5 may have other functions in the cell besides those associated with the Dsc E3 ligase.

Introduction

Our lab discovered the Dsc E3 ligase during a series of experiments designed to find genes required for sterol regulatory element-binding protein (SREBP) cleavage in the fission yeast *Schizosaccharomyces pombe* (Stewart, Nwosu, et al. 2011, Stewart, Lloyd, et al. 2012). This membrane-bound protein complex contains five members, aptly named defective for SREBP cleavage (Dsc) proteins Dsc1-Dsc5. Previously, we reported that the Dsc E3 ligase localizes to the Golgi, based on microscopy studies that localized Dsc2. Further work to show the connectivity of the Dsc E3 ligase complex determined that Dsc2 was centrally localized, connected in one direction to Dsc1, Dsc3, and Dsc4, and connected in the other direction to Dsc5.

Unpublished data in our lab started to indicate that Dsc5 might have distinct roles in the cell that are separate from those of the entire Dsc E3 ligase (Lloyd, Raychaudhuri and Espenshade 2013). Studies that looked at complex formation by blue native PAGE showed that free Dsc5 existed in wild-type cells, and this pool of Dsc5 increased in a *dsc2Δ* strain (Lloyd, Raychaudhuri and Espenshade 2013). In looking at the requirement of *dsc5* in SREBP cleavage, a *dsc5Δ* strain showed a more mild cleavage defect for both Sre1 and Sre2 proteins (Cheung and Espenshade 2013). Supported by the fact that Dsc5 was not as integrated in the complex as other members because it was only connected

through Dsc2, I wondered if Dsc5 could be localized differently than the localization that had been determined for Dsc2. To address this question, I took a fluorescence microscopy approach to look at the localization of Dsc5 in the cell.

Results

To determine the localization of Dsc5, I used a strain that expressed Dsc5-GFP from the endogenous promoter of the *dsc5* gene (Figure 1). I crossed this strain to strains that had ER- or Golgi-localized proteins labeled with mCherry (Ost1 and Anp1, respectively) (Vjestica, Tang and Oliferenko 2008), then used fluorescence confocal microscopy to co-localize the GFP- and mCherry-labeled proteins. I found that Dsc5 had partial co-localization with both the ER marker Ost1 and the Golgi marker Anp1 (Figure 2). Anp1 marks the *cis* Golgi, and shows partial colocalization with Dsc2 (Stewart, Nwosu, et al. 2011).

I also performed immunofluorescence with the anti-Dsc5 antibody (Figure 2). As demonstrated by Figure 1, this antibody is highly specific for Dsc5. The immunofluorsence on a *dsc5Δ* strain showed a high amount of non-specific staining by the anti-Dsc5 antibody. Taking this background into account, it looked as though the immunofluorescence on the wild-type strain confirmed the localization that was determined using the Dsc5-GFP strain. Taken together, these results indicate that Dsc5 has a localization that is unique from the rest of the Dsc E3 ligase components, and is partially localized to both the ER and the Golgi.

Discussion

The Dsc E3 ligase complex is a tightly-associated, membrane-bound complex of proteins. The components, Dsc1-Dsc5, have stable physical interactions that have been determined by mass spectrometry, co-immunoprecipitation, and blue native PAGE (Stewart, Nwosu, et al. 2011, Stewart, Lloyd, et al. 2012, Lloyd, Raychaudhuri and Espenshade 2013, Todd, et al. 2006). During the course of studying Dsc5, it became apparent that this member of the Dsc E3 ligase may have distinct functions, and might exist outside of the complex. Using fluorescence microscopy, I confirmed that Dsc5

partially co-localizes to the Golgi with the rest of the Dsc E3 ligase, but also shows partial localization to the ER.

Dsc5 has two homologs in *S. cerevisiae*, Ubx2 and Ubx3. Althought these paralogous proteins are similar to each other, it was shown that only Ubx3 was functionally related to Dsc5 (Ryan, et al. 2012). Knowing the localization of Dsc5 may be able to further inform its relationship to Ubx2 and Ubx3, as preliminary data from our lab suggests that Ubx2 and Ubx3 do not share the same localization (Zongtian Tong, unpublished data). It is possible that Dsc5 has conserved functions related to Ubx2 in addition to those shown for Ubx3.

Dsc5 is also conserved in mammalian cells where its homolog is called UBXD8. A recent paper showed that UBXD8 contained domains that allowed it to polymerize in response to long chain unsaturated fatty acids, and that UBXD8 localized to lipid droplets (Kim, et al. 2013). Initial experiments I performed to localize Dsc5 in the presence of excess oleate were unsuccessful, due to a poor combination of reagents. I attempted to treat cells with oleic acid coupled to BSA, then stain the cells with FM4-46 dye and allow the dyed membrane to be internalized and mark any internal lipid structures. I used the Dsc5-GFP strain to perform live-cell imaging and co-localize the Dsc5 with the FM4-64 stained structures. Unfortuantely, FM4-64 is flurorescent under a 488 nm laser, so I was unable to distinguish the dyed membrane from the Dsc5-GFP protein. Nevertheless, this is an exciting potential role for Dsc5 outside of the Dsc E3 ligase complex, and should be investigated.

Materials and Methods

Strains, Media, and Antibodies

Standard YES medium was used to grow all strains. Strains used in this study are listed in Table 1. Anti-Dsc5 antibody was generated by Covance, Inc., using a fragment of the Dsc5 protein (aa 251-427). Anti-rabbit IgG-Alexa 594 antibody was purchased from Invitrogen.

Fluorescence Confocal Microscopy

Images were taken in the Johns Hopkins Microscope Facility using a 3i Spinning Disk Confocal Microscope (more information on this microscope is detailed in Chapter 2 of this thesis). A 100 X oil objective and 1.0 optivar was used for all images. GFP was imaged using a 488 nm laser and mCherry was imaged using a 562 nm laser. Cells were prepared for the microscope slide as follows: first, cells were grown to log phase in rich medium, then 1 mL of culture was pelleted and the resulting supernatent was discarded by dumping in the sink (not aspiration). Cells were resuspended in residual liquid, and 2.5 µL was plated on a 2% agarose pad on a slide. Coverslips were added and slides were sealed with clear nail polish.

Immunofluorescence

Immunofluorescence was performed using a protocol adapted from the Nurse lab Pombe handbook. Slides were prepared as for live-cell microscopy, and a 100 X oil objective, 1.0 optivar was also used on the 3i spinning disk confocal microscope. The anti-Dsc5 antibody was used as the primary antibody (not affinity-purified) at a concentration of 1:500.

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Figure Legends

Figure 1: Dsc5-GFP has normal expression as determined via western blot

Western blot with anti-Dsc5 antibody. The Dsc5-GFP strain expresses a fusion protein from the endogenous *dsc5* promoter. The level of the Dsc5-GFP fusion protein (lane 2) is roughly equivalent to the endogenous Dsc5 protein (lane 1), indicating that the fusion protein was stable in the cell.

Figure 2: Live-cell images of Dsc5-GFP show co-localization with ER and Golgi markers

A) Co-localization of Dsc5 and the ER marker Ost1. Two sections are shown, each taken from a different area of the microscope slide. The merged image shows the Dsc5 protein partically co-localizes with the Ost1 protein in sections from confocal microscopy of live

cells. B) Co-localization of Dsc5 and the *cis*-Golgi marker Anp1. Two sections are shown, each taken from a different area of the microscope slide. The merged images shows the Dsc5 protein partially co-localizes with the Anp1 protein in sections from confocal microscopy of live cells.

Figure 3: Immunofluorescence images of Dsc5 confirm localization from live-cell images

Panel (A) shows wild-type *S. pombe* stained with the anti-Rabbit IgG-Alexa 594 antibody alone to show non-specific staining by the secondary antibody. Panel (B) shows the *dsc5Δ* strain stained with both the anti-Dsc5 primary and Alexa 594 secondary antibodies, demonstrating non-specific reactivity of the primary antibody. Panel (C) shows wild-type *S. pombe* stained with anti-Dsc5 primary and Alexa 594 secondary. The staining pattern resembles the Dsc5-GFP localization observed in Figure 2, confirming that the endogenous and tagged proteins have the same localization. The staining pattern also confirms that Dsc5 is partially localized in the ER, since perinuclear staining is clearly observed, but is also localized to the Golgi, as punctate structures are also present.

Tables

Table 1: Strains used in this study

Strain	Genotype	Source
KGY425	Wild-type	ATCC
YCN85	*dsc5Δ*	Stewart 2012
YCN69	*dsc5-GFP*	This study
DJY029	*dsc5-GFP,ost1-mCherry*	This study
DJY006	*dsc5-GFP, anp1-mCherry*	This study

Figure 1

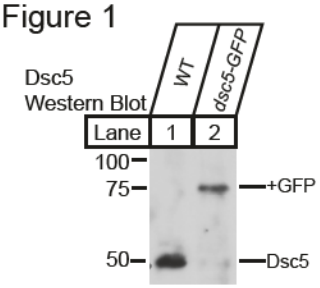
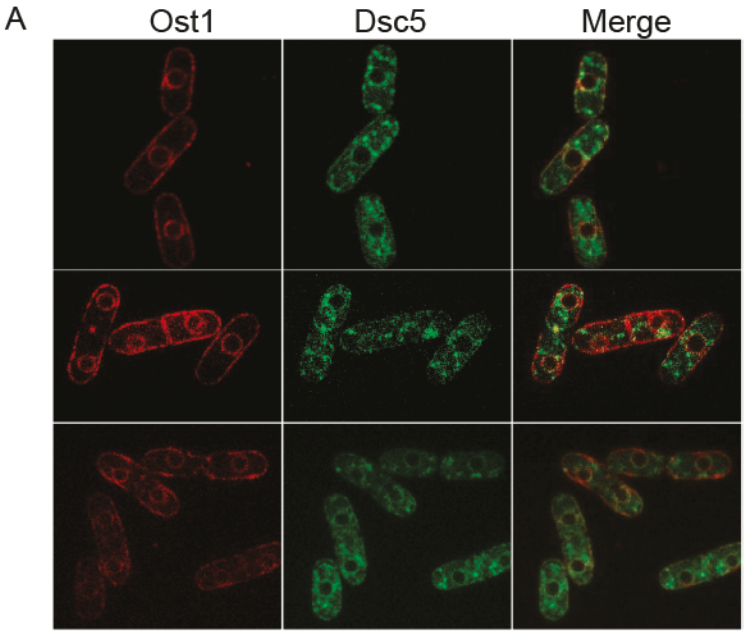


Figure 2



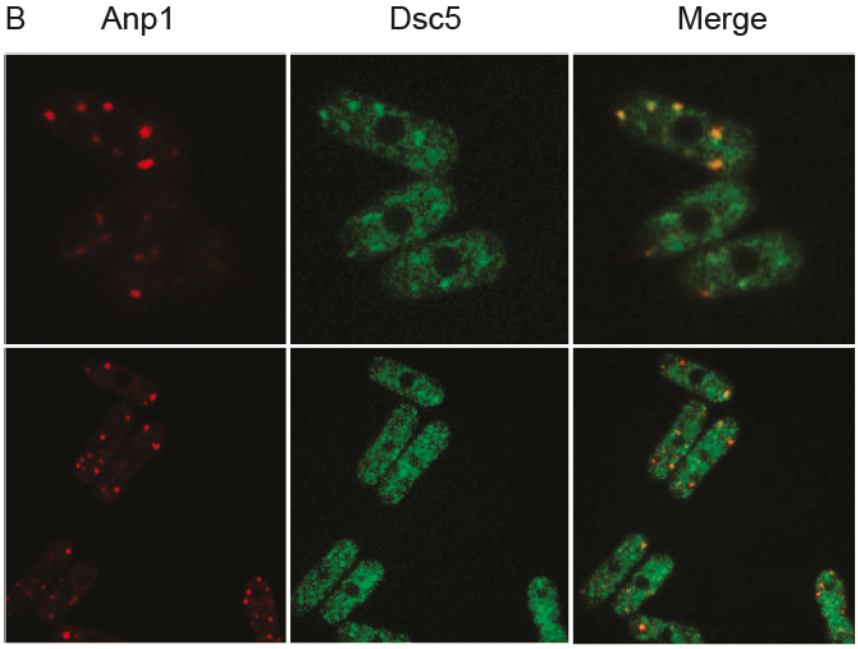
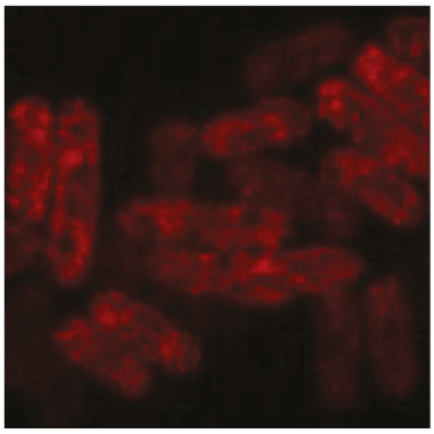


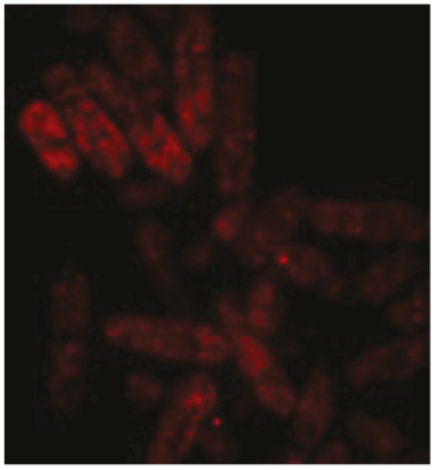
Figure 3

A



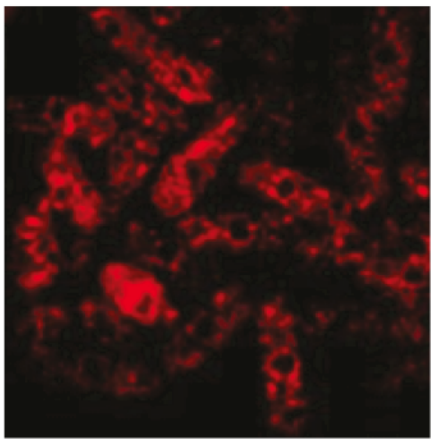
Strain: WT
Primary: none
Secondary: anti-Rabbit IgG- Alexa 594

B



Strain: *dsc5*Δ
Primary: anti-Dsc5
Secondary: anti-Rabbit IgG- Alexa 594

C



Strain: WT
Primary: anti-Dsc5
Secondary: anti-Rabbit IgG- Alexa 594

Curriculum Vitae for
Diedre J. Ribbens, PhD

Formerly Diedre J. Johnson
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Educational History

PhD	2013	Program in Biochemistry, Cell and Molecular Biology	Johns Hopkins School of Medicine Mentor: Peter J. Espenshade, PhD
B.S	2009	Genetics, Cell Biology, and Development (Honors) Development (Honors)	University of Minnesota Mentor: Clifford J. Steer, M.D.
	2009	Microbiology	University of Minnesota Mentor: Dana A. Davis, PhD

Other Professional Experience

Senior Technical Editor	2013-Present	Contracted by Edanz Group China
Science Writer	2012-Present	Freelance/Self-employed
Research Rotation	2009	Lab of Steven Claypool, PhD Johns Hopkins School of Medicine Department of Physiology

Research Rotation	2009	Lab of Joel Pomerantz, PhD Johns Hopkins School of Medicine Department of Biological Chemistry

Academic or Other Honors at Johns Hopkins School of Medicine

Oral Presentation Award, 1st Place, BCMB Retreat	October 2012
Department of Cell Biology Student Travel Award	July 2012
Graduate Student Association Travel Award	July 2012

Publications, Peer-reviewed

Wolf, J.M., **Johnson, D.J**., Chmielewski, D., and Davis, D.A. (2010) The *Candida albicans* ESCRT Pathway Makes Rim101-Dependent and -Independent Contributions to Pathogenesis. *Eukaryotic Cell* 9(8): 1203-1215. PMICID: PMC2918940

Publications, Non-peer-reviewed

"An Enduring Accomplishment: The Founding of the Howard Hughes Medical Institute." *BCMB News.* November 2012. http://bcmbnews.com/2012/11/02/an-enduring-accomplishment-the-founding-of-the-howard-hughes-medical-institute/#more-672

"Thematic minireview series centers of circular proteins." *ASBMB Today.* September 2012. http://www.asbmb.org/asbmbtoday/asbmbtoday_article.aspx?id=17887

"Yeast Genetics 2012: Beyond Beer, Bread, and Wine." *The GSA Reporter.* Fall 2012 (p. 18-19) http://www.genetics-gsa.org/pdf/Fall2012Web.pdf

Posters & Abstracts

"A Rhomboid Protease Involved in SREBP Processing in *Schizosaccharomyces pombe*." Abstract for Platform Session, Yeast Genetics and Molecular Biology Meeting. Sponsored by the Genetics Society of America. Princeton University, July 31-August 5 2012.

Service & Leadership

2012-2013 Student Course Director, Effective Science Communication

Scheduled guest lecturers and gave several lectures for a course covering topics in science communication. Course material included grammar, language, and style; manuscript writing; writing grant proposals; writing abstracts; making tables and figures; science communication through social media; communicating science to the lay public; and communicating science to policy makers.

2012-2013 Creator & Editor-in-chief, BCMB News

Created a monthly newsletter for the BCMB program, published in an online format. Solicited and edited submissions from students, faculty, and alumni of the BCMB Program. Learned to use the Wordpress publishing platform.

2012-2013 Student Representative, Johns Hopkins University MSCHE Reaccreditation Self-Study

Represented the interests of graduate students on a university-wide committee to organize and implement the self-study process required for accreditation. The Middle States Commission on Higher Education (MSCHE) accrediting body interacted with this committee over the course of several years, working with us to develop an accurate and complete self-study. I directly assisted in generating material for the self-study report and soliciting input from other students and trainees.

2011-2013 Organizer, BCMB Student Research Colloquium

Along with two other organizers, I helped schedule student speakers for each monthly seminar luncheon.

2011-2013 BCMB Policy Committee, student representative

I served a two-year term on the BCMB Policy committee. I was elected to the position by my peers, and contributed to important issues. My main project that instituted change was a survey of

current students about a particular required course. The student body felt the course was not serving their needs, so I designed and distributed a survey to gather data and opnions, and presented this to the faculty on the policy committee. They voted to change the course to better meet students' needs.

2013 Invited Speaker and Research Supervisor, Junior Biomedical Scholars

The Junior Biomedical Scholars are high school juniors and seniors from Paul Lawrence Dunbar High School in Baltimore, near the Johns Hopkins Medical campus. I spent several weekends talking to them about my journey to becoming a scientist, and helping them with their own research projects for an upcoming science fair.

2013 Private Tutor, Writing & Language Arts

I tutored a 7th-grade student who was having difficulties expressing his thoughts through writing. I worked with him for several months on the basics of how to write an essay, and taught him grammar and style rules as appropriate for his grade.

2012 Teaching Assistant, Effective Science Communication

I assisted the student course director in giving lectures, grading assignments, and arranging guest speakers.

2011 Teaching Assistant, Genetics & Genomics

As teaching assistant for the Yeast Genetics section, I wrote a "problem set" homework assignment that covered the material taught by Dr. Susan Michaelis. I then led the students in a review session that clarified information from Dr. Michaelis' lectures, and assisted them in working through their problem set. I also helped students review for the final exam, wrote their exam questions on the yeast genetics section, and graded both the problem set and the exam. The large class size (85 students) made this a unique challenge, because the students had varied levels of background knowledge on the subject.